We highlight previous incompletely understood cell biology data in the STAT3 signaling field with respect to interleukin-6 (IL-6)-induced activation of this transcription factor in hepatoma cells to generate cytoplasmic and nuclear STAT3 bodies. We provide a novel re-interpretation of the previous observations. We show that IL-6-induced GFP-STAT3/PY-STAT3 cytoplasmic and nuclear bodies represent phase-separated biomolecular condensates. These structures represent examples of a cytokine-induced phase transition which occurs within 10–15 min of exposure to the cytokine, and which was Tyr phosphorylation dependent. Evidence that these IL-6-induced cytoplasmic and nuclear GFP-STAT3 bodies in live cells represented phase-separated condensates came from the observation that 1,6-hexanediol caused their disassembly within 30–60 seconds. Moreover, these STAT3 condensates also showed rapid tonicity-driven phase transitions – disassembly under hypotonic conditions and reassembly when cells were returned to isotonic medium. That STAT3 condensates were rapidly disassembled in hypotonic buffer commonly used for cell fractionation points to a limitation of studies of STAT3 biochemistry using hypotonic swelling and mechanical breakage. Overall, the new data help reinterpret IL-6-induced cytoplasmic and nuclear GFP-STAT3 bodies as phase-separated biomolecular condensates, and bring the concept of membrane-less organelles to the cytokine-induced STAT transcription factor field and cancer cell biology.

**Key words:** interleukin-6, cytokine-inducible biomolecular condensates, cytoplasmic STAT3, nuclear STAT3 bodies, 1,6-hexanediol, liquid-like condensates, Tyr phosphorylation-driven phase transitions, tonicity-driven phase transitions, cytokine signaling.
Cytokines and STAT3 in cancer cell biology

It is now well established that a large number of cytokines that signal to the cell interior by activating the transcription factor STAT3 are directly involved in the processes of carcinogenesis and metastasis [16, 17]. This is especially true in the context of the local tumor microenvironment, where interleukin-6 (IL-6) and activated STAT3 play key roles in cancer progression [16, 17]. Extensive studies of the IL-6/STAT3 pathway in hepatoma cell lines (Hep3B, HepG2), and other cell types, using GFP-STAT3, have revealed the presence of IL-6-induced cytoplasmic and nuclear “bodies” [18–20].

In 2003, Herrmann et al. called attention to GFP-STAT3 nuclear bodies in IL-6-treated Hep3B cells [18]. The appearance of nuclear GFP-STAT3 bodies required IL-6 induction; these contained Tyr-P-STAT3 (PY-STAT3), and, by FRAP, were observed to contain a pool of GFP-STAT3 that was readily mobile [18]. The IL-6-induced nuclear STAT3 bodies also contained the CREB-binding protein (CBP) and histone H4, which are markers for transcriptionally active chromatin. In contrast, these STAT3 nuclear bodies were distinct from the promyelocytic leukemia oncoprotein (PML) bodies. Parenthetically, PML bodies also are now known to be phase-separated condensates [1–5].

In 2007, we called attention to GFP-STAT3 cytoplasmic bodies (we called these “STAT3 sequestering endosomes”) in IL-6-treated Hep3B cells [19]. These GFP-STAT3 bodies required IL-6 induction, contained PY-STAT3, required the Y705 tyrosine in STAT3, and were transient in that these disappeared in 2–3 hours. Moreover, their appearance was blocked by phosphorylation inhibitors such as genistein, staurosporine and indirubin E804, and by the microtubule inhibitor nocodazole [19]. These IL-6-inducible cytoplasmic STAT3 bodies were observed irrespective of the fluorescent tag used (GFP, YFP or DsRed) [19, 20]. Curiously, we were unable to associate any of the known endosome markers with these IL-6-induced cytoplasmic GFP-STAT3 sequestering structures by co-localization assays using immunofluorescence techniques (data not shown). Moreover, extensive attempts to obtain subcellular fractions enriched in such STAT3 sequestering “endosomes” were unsuccessful (data not shown). Critically, this is now no longer surprising in that we note that all such cell fractionation experiments in our hands began with hypotonic swelling of IL-6-induced Hep3B cells followed by Dounce mechanical breakage of the cells (see Fig. 5 below).

Interleukin-6-induced cytoplasmic and nuclear GFP-STAT3 bodies in Huh7 hepatoma cells

We have extended these previous GFP-STAT3 observations of nuclear bodies from the Heinrich laboratory [18] and of cytoplasmic bodies from our laboratory [19, 20], to Huh7 hepatoma cells. Figure 1 illustrates examples of untreated GFP-MxA expressing Huh7 cells as well as those exposed to IL-6 for 15–20 min. The latter cells showed extensive development of cytoplasmic and nuclear GFP-STAT3 bodies.

Figure 2A summarizes the rapidly inducible nature of the appearance of cytoplasmic GFP-STAT3 bodies of IL-6-treated Hep3B cells. The cytoplasmic bodies appeared by 10–15 min [19]. Moreover, Figure 2B summarizes the presence of PY-STAT3 in such cytoplasmic (and nuclear) GFP-STAT3 bodies [19]. We note that these GFP-STAT3 structures, cytoplasmic and nuclear, were seen only in cells stimulated with cytokine.

Similar to observations with condensates of cGAS [6] and of MxA [15], the data in Figure 3A (from 2007) show that cytoplasmic GFP-STAT3 bodies were resistant to digitonin [19]. Moreover, Figure 3B shows that even native endogenous STAT3/PY-STAT3 formed punctate structures in the cytoplasm of IL-6-treated Hep3B cells that resisted digitonin, but were disassembled by Brij-58 [19].

Interleukin-6-induced cytoplasmic and nuclear bodies were tonicity-regulated biomolecular condensates

We applied our recent insights into the structure of GFP-MxA condensates in Huh7 cells [14, 15] to the IL-6-induced GFP-STAT3 cytoplasmic and nuclear bodies. Figure 4 shows three independent experiments in which IL-6-induced GFP-STAT3 cytoplasmic and nuclear bodies were disassembled in less than 1 min by exposure to hexanediol. These data provide evidence that the cytoplasmic and nuclear GFP-STAT3 bodies comprised phase-separated biomolecular condensates with liquid-like properties. Even more striking was the discovery summarized in Figure 5 that the integrity of both the cytoplasmic and nuclear bodies was regulated by the toxicity of the culture medium. A switch to hypotonic ELB medium led to disassembly of both cytoplasmic and nuclear GFP-STAT3 bodies within 1–3 min. Re-exposure to isotonic medium led to reassembly of cytoplasmic and nuclear GFP-STAT3 into discrete structures — but different from the starting structures. These observations recapitulate the tonicity-driven disassembly and reassembly of GFP-MxA in Huh7 cells observed by us [14, 15] and suggest that cytoplasmic “crowding” [4, 8] may be a likely mechanism regulating these phase separations.

Practical implications

The discovery that the standard hypotonic buffer used by many investigators for cell swelling (as is typically the
first step prior to mechanical cell breakage in cell-fractionation protocols) led to rapid and marked disassembly of IL-6-induced cytoplasmic and nuclear GFP-STAT3 condensates (Fig. 5) has implications for interpreting hundreds of studies of the biochemistry of STAT3. We show by the data in Figure 5 that hypotonic cell swelling introduces a hitherto unrecognized limitation into all such studies – there occurs rapid disassembly of cytoplasmic protein condensates under such fractionation conditions.

The recognition that IL-6-induced GFP-STAT3 existed, at least in part, in the cytoplasm and nucleus in biomolecular condensates highlights the possibility of cross-talk between STAT3 and other signaling pathways. Herrmann et al., already in 2003 [18], pointed to the co-association of PY-STAT3 with CBP and histone H4 in IL-6-induced nuclear bodies (which we now identify as “biomolecular condensates”). In line with our previous data on MxA condensates which included co-condensation with cyclic GMP-AMP synthase (cGAS) [15], we suggest that cytoplasmic condensates, such as of GFP-STAT3, might also allow for the physical/spatial segregation of signaling pathways in the cytoplasm as well as cross-talk with other novel signaling mechanisms.

“STAT-masking”: another possible example of IL-6-induced wt p53-dependent STAT3 phase transition in hepatoma cells

In 1997–1998 we reported the curious phenomenon wherein bulk cytoplasmic STAT3 and STAT5 in Hep3B cells expressing wild-type p53 and then treated with IL-6 transiently lost their ability to be detected by immunofluorescence methods, even though there was no loss of the respective proteins [21, 22]. This phenomenon, dubbed “STAT-masking,” was evident in less than 30 min after IL-6 exposure and lasted 2–3 hours [21, 22]. IL-6-induced STAT-masking was selective in that it was observed for STAT3 and STAT5, but not STAT1. STAT-masking required IL-6-induced Tyr phosphorylation as well as wild-type p53 expression and protein synthesis for at least 6–8 hours prior to its successful elicitation [21, 22]. This loss of immunoreactivity of STAT3 and STAT5 may represent a novel bulk phase transition mechanism dependent upon a p53-induced cellular protein. That exposure of Hep3B cells to IL-6 leads to the development of large (1-2 MDa) complexes of GFP-STAT3 has been inferred from fluorescence correlation spectroscopy of the cytoplasm of live cells [23].

Fig. 1. Interleukin (IL)-6-induced cytoplasmic and nuclear GFP-STAT3 bodies in Huh7 hepatoma cells. Just subconfluent cultures of Huh7 cells in 35 mm plates that had been transiently transfected with the pGFP-STAT3 expression vector one day earlier were imaged using live-cell microscopy without IL-6 exposure or after exposure to IL-6 (20 ng/ml) for 15–20 min using methods outlined by Xu et al. [19]. Nuc – nucleus, Cytopl – cytoplasm. Scale bar = 25 µm
Fig. 2. Association of GFP-STAT3/PY-STAT3 with cytoplasmic structures in interleukin-6 (IL-6)-treated Hep3B hepatoma cells (this figure is an abbreviated version of Figure 1 of Xu et al. [19]).

A) Hep3B cells cultured in 6-well plates were transfected with the pGFP-STAT3 construct and imaged 20 hours later using live-cell confocal microscopy. IL-6 (25 ng/ml final concentration) was added immediately after the "0 minutes" frame and the cells were imaged at 15 seconds intervals for the next 18 min. Selected frames from this time-lapse sequence at indicated times in minutes are illustrated.

B and C) Hep3B cultures co-transfected with pGFP-STAT3 construct one day earlier were treated with IL-6 for 30 min, fixed with paraformaldehyde and immunostained with anti-PY-STAT3 pAb. The two panels illustrate data from two independent experiments. All scale bars = 25 µm.

Fig. 3. Interleukin-6 (IL-6)-induced GFP-STAT3 and endogenous PY-STAT3-containing cytoplasmic bodies in Hep3B hepatoma cells were resistant to digitonin (this figure is an abbreviated version of Figure 2 of Xu et al. [19]).

A) Hep3B cultures transfected with the pGFP-STAT3 construct were first treated with IL-6 for 30 min in the presence of LysoTracker (in red) with GFP-STAT3 in green. These were then sequentially imaged upon treatment with digitonin (50 µg/ml) as indicated.

B) Replicate Hep3B cultures were exposed to IL-6 for 30 min and then sequentially to digitonin (50 µg/ml) in ice-cold 0.25 M sucrose/phosphate-buffered saline (sucrose buffer) or Brij58 (0.5% v/v in sucrose buffer), fixed with paraformaldehyde and immunostained for PY-STAT3. All scale bars = 25 µm.
Conclusions

We provide a novel reinterpretation of previous incompletely understood cell biology data in the STAT3 signaling field with respect to IL-6-induced activation of this transcription factor in hepatoma cells and the formation of cytoplasmic and nuclear STAT3 bodies. We now recognize that the IL-6-induced GFP-STAT3/PY-STAT3 cytoplasmic and nuclear bodies represent phase-separated biomolecular condensates which disassembled rapidly in the presence of hexanediol. This IL-6-induced generation of these cytoplasmic and nuclear STAT3 bodies represents an example of a cytokine-triggered phase transition which is Tyr phosphorylation dependent and which occurred in less than 10–15 min. Moreover, we observed that these STAT3 condensates showed rapid tonicity-driven phase transitions. The new insights extend the concepts of phase-separated biomolecular condensates to the cytokine-induced activation of STAT transcription factors in cancer cell biology, and point to a novel underlying biochemistry.

Acknowledgements

I thank Dr. Joseph D. Etlinger for insightful discussions, and Ms. Jenna Westley for technical assistance.

This work was supported, in part, by funding from the New York Medical College, and by personal funds of PBS. The author declares no conflict of interest.
Fig. 5. Culture-medium tonicity drives disassembly and reassembly of interleukin-6 (IL-6)-induced GFP-STAT3 cytoplasmic and nuclear condensates in Huh7 cells. Huh7 cultures in 35 mm plates transfected with pGFP-STAT3 vector one day earlier were left untreated with cytokine (upper row) or treated with IL-6 (20 ng/ml) for 15–20 min (lower row) and imaged in isotonic medium (DMEM/PBS; approximately 325 milliosmolar), then switched to hypotonic medium (erythrocyte lysis buffer (ELB), approximately 40–50 milliosmolar) for 3 min, and then back to isotonic medium (DMEM/PBS) as indicated. Scale bar = 25 µm.

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Address for correspondence
Pravin B. Sehgal
Department of Cell Biology and Anatomy
New York Medical College
Valhalla, NY 10595, USA
e-mail: pravin_sehgal@nymc.edu

Submitted: 13.01.2019
Accepted: 21.01.2019