Correspondence

Tumor Exosome-Mediated MDSC Activation

To the Editor-in-Chief:

In a recent issue of *The American Journal of Pathology*, Xiang et al[1] published their results concerning the effects of tumor-derived exosomes on myeloid-derived suppressor cell (MDSC) biology. They compared the biological effects of exosomes derived from *in vitro* cultured B16 tumor cells (termed *C-exo* for culture exosome) and exosomes derived from *in vivo* grown B16 tumor (termed *P-exo* for primary exosomes). They reported that P-exo induce Toll-like receptor 2 (TLR2)–independent MDSC activation and expansion, whereas C-exo activate and expand MDSC in a TLR2-dependent manner. These data are in contrast with our own recent article demonstrating MDSC activation through TLR2 ligation by heat shock protein 72 (Hsp72) expressed on exosomes from CT26 tumors.[2] In their discussion, Xiang et al[1] propose a hypothesis to explain this discrepancy between C-exo and P-exo. Here, we provide our point of view about this difference.

Xiang et al[1] propose that P-exo harbor a TLR2-independent mechanism of MDSC activation that is different from C-exo. This is probably true. Indeed, in another study from the same group, Xiang et al[3] provided evidence that TS/A tumor cell lines produce exosomes containing prostaglandin E_2 (PGE_2), and it has previously been shown that PGE_2 alone is sufficient to induce MDSC expansion and activation in a TLR2-independent manner.[4] However, in the models used in our study, we provide evidence that there is no detectable PGE_2 in tumor-derived exosomes. Xiang et al[1] assert that our work demonstrated that "tumor exosomes trigger MDSC expansion via activation of STAT3." However, this assumption is false. In our model, we demonstrated that there are indeed two distinct signals: tumor-derived exosomes account for MDSC activation (STAT3 phosphorylation and interleukin 6 secretion), whereas tumor-derived soluble factors [namely, granulocyte-macrophage colony-stimulating factor (GM-CSF)] are responsible for expansion. In the EL4 model, we observed that tumors growing in TLR2$^{-/-}$ mice induced MDSC expansion but not MDSC activation compared with wild-type mice. Thus, we can exclude the *in vitro* effect hypothesized by Xiang et al,[1] because, in this *in vivo* setting, we observed proliferation dissociated from activation. In the model by Xiang et al,

for unknown reasons, C-exo induced both STAT3 activation and expansion of MDSC; however, we never observed proliferation of MDSC cultured with exosomes alone. Moreover, Xiang et al already reported that the expansion and proliferation of MDSC in their model may be related to the presence of PGE_2. Indeed, we also observed that PGE_2 alone could trigger MDSC expansion and proliferation, so we could postulate that exosomes may have contained PGE_2 in the model of Xiang et al, whereas we could not detect PGE_2 in exosomes from our own models. These data may explain the discrepancy between our works.

Furthermore, the preparation of the P-exo raises some methodological concerns. They are prepared from isolated *in vivo* grown tumors, with less than 5 *in vitro* passages. Many nontumoral cells, such as myeloid cells or fibroblasts, might be present in the preparation. These cell contaminants could be responsible for the production of PGE_2-containing exosomes because it is well known that tumor-infiltrating macrophages may produce PGE_2[5] and exosomes.[6]

Xiang et al[1] also propose that the difference between C-exo and P-exo is based on the passage number of *in vitro* cultured cells. We work with cell lines obtained from the American Type Culture Collection and used at passage numbers less than 15 to 20, so we assume the derivation from the parental cell line is minimal. Xiang et al also proposed that mycoplasma contamination could be responsible for the TLR2-dependent activation signal, which would be eliminated by the *in vivo* passage of the tumor. In our study, we routinely tested for mycoplasma contamination, but Xiang et al suspect that the available tests are insufficient. However, in our study, we demonstrated that exosomes derived from mycoplasma-free Hsp72-silenced CT26 were unable to activate MDSC, whereas mycoplasma-free mock CT26-derived exosomes activated MDSC. Further, *in vivo* Hsp72-silenced tumors were unable to induce STAT3 activation of MDSC, thus supporting the premise that the Hsp72-TLR2-MyD88 pathway is vital in our model. In our opinion, this clearly excludes concerns about a potential effect of mycoplasma contamination because the Hsp72 status alone would not be a determinant otherwise.

In summary, we propose that the discrepancy identified by Xiang et al[1] between C-exo and P-exo may

actually rely on the presence of tumor exosome–associated PGE_2 produced by tumor or contaminant cells, which bypass the Hsp72-TLR2-MyD88 signal.

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Author's Reply:

In comparing the data published by our group[1] with that published by Chalmin et al,[2] the major discrepancies are that i) they did not detect PGE_2 on the tumor exosomes purified from the same type of tumor cells, whereas we could detect PGE_2, and ii) our data do not support a major role for TLR2 in tumor exosomes in mediating either activation or expansion of MDSCs.

Regarding the first point, we still believe that our data closely represent what occurs *in vivo* because tumor exosomes play a role in the induction of chronic inflammation,[3,7–15] and PGE_2 released from different types of cells, including tumor cells, has the capacity to bind tumor exosomes. Therefore, PGE_2 does not have to enter exosomes before they are released from the cell because association could occur after PGE_2 is released from many types of cells. In the tumor microenvironment, large numbers of nontumor and tumor cells release PGE_2, which subsequently binds to exosomes. This is not contamination as suggested by Ghiringhelli and colleagues. In fact, our published data of exosomes released from *ex vivo* cultured tumor cells[1] closely represent what occurs in the tumor microenvironment. Whether PGE_2 is released from tumor cells or nontumor cells should be further tested. However, the key point for designing future therapeutic strategies is that our data provide valuable information as a close reflection of what takes place *in vivo*.

Second, when hypothesizing that tumor exosomes play a role in immune suppression through the expansion of MDSCs, we believe it is more convincing that tumor cells have contact with immune cells, including T cells, as demonstrated in our report.[1] It is conceivable that exosomes released from *in vitro* cultured tumor cells do not have to actively sort the molecules into exosomes for immune suppression because the immune suppression is initiated after long-term *in vitro* culture. In contrast, the exosomes released from tumor tissue or after *ex vivo* culture with immune cells are expected to exhibit more potent immunosuppressive function because tumor cells possess multiple immune escape strategies. Therefore, we believe that after tumor cells communicate with immune cells, including infiltrated T cells, these tumor cells develop immune escape mechanisms to avoid the host TLR2-mediated innate immune response. Whether tumor exosomes or GM-CSF alone is sufficient to cause the expansion of MDSCs is difficult to conclude, because in our systems[1] bone marrow precursors cells were cultured in the presence of GM-CSF and tumor exosomes.

Finally, regardless of the strategies to eliminate the possibility of mycoplasm contamination, one should not exclude the possibility of unidentified species of mycoplasma contamination because TLR-mediated signaling is easily stimulated even with low levels of an appropriate ligand. Collectively, our data support the notion that tumor cells should be reisolated frequently from an immune-competent host for examining tumor exosome-mediated immunosuppression. Otherwise, the data generated from *in vitro* cultured cells are less meaningful for clinical translational research.

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