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Elimination of fluorescent protein immunogenicity permits modeling of metastasis in immune-competent settings

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Transplantation models allow one to faithfully recapitulate the clinical course of metastatic disease, functionally screen for regulators of dormancy and metastasis, and conduct gold-standard assessment of metastasis-initiating potential (Quintana et al., 2008). Historically, these studies have been conducted in immune-compromised settings. Given the need to understand how tumor-stroma-immune axes influence progression and augment response to radiotherapies, chemotherapies, and immunotherapies, such studies are now conducted in immune-competent settings with greater frequency. Here, we have run into a significant issue.

Quantifying metastatic progression in mice commonly requires the introduction of proteins such as those derived from a firefly (i.e., firefly luciferase, ffLUC) and/or a jellyfish (e.g., green fluorescent protein, GFP) to monitor progression and metastasis *ex vivo* and to quantify tumor burden in tissue sections. This presents a complication in that mice mount an immune response against these foreign antigens (Gambotto et al., 2000; Han et al., 2008; Limberis et al., 2009). Indeed, despite immunologists using GFP as a model antigen (Agudo et al., 2015; Malhotra et al., 2016), GFP and its derivatives are employed routinely in metastasis studies but are rarely controlled for. It remains unclear how their expression affects metastatic progression, the degree to which an immune response to these "neo-antigens" should be attenuated, and how to achieve the level of attenuation required for unadulterated progression.

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Declaration of interests

The authors declare no competing interests.

To address these questions, we employed the widely used mammary tumor line 4T1, which is highly metastatic. We orthotopically implanted 4T1 cells that express GFP at a level that is necessary to distinguish single, disseminated tumor cells and metastases in distant sites ("4T1-high"). We found that, in contrast to immune-compromised NOD-SCID mice, 4T1-high cells began to be rejected ~11 days after inoculation of syngeneic BALB/c hosts, lost GFP expression, and very rarely formed lung metastases (Figure S1A–F). This was accompanied by a robust GFP-specific $CD8^+$ T cell response (Figure S1D) as measured via ImmunoSpot assay.

In an attempt to mitigate the GFP-specific $CD8^+$ T cell response, we tested whether simply lowering tumoral GFP levels was sufficient to allow for progression and metastasis. We generated an additional lentivirally transduced line ("4T1-low") that expressed ~4% the GFP-level of its 4T1-high counterpart (Figure S1A). Notably, 4T1-low cells generated tumors of similar volume and GFP level in immune-compromised and in immune-competent mice (Figure S1B–C). In line with these data, 4T1-low cells generated a muted but non-zero GFP-specific $CD8^+$ T cell response compared to 4T1-high cells (Figure S1D). Despite this, GFP^+ metastases did not eventuate from 4T1-low cells (Figure S1E), even though 4T1-low and 4T1-high cells exhibited similar metastatic potential in NOD-SCID mice as assessed by measuring the number of phospho-histone H3-positive ($pH3^+$) lung metastases (Figure S1F). These data suggest that attenuation of the GFP-directed $CD8^+$ T cell response is insufficient for tissue colonization of detectably tagged cells in immune-competent settings.

We next considered modulation of host immunity as a potential solution, based on the reasoning that, in transgenics, expression of antigens like GFP and ffLUC since birth might enable progression and maintenance of tumoral ffLUC/GFP expression. This is the idea behind several stated tolerized models (Aoyama et al., 2018; Day et al., 2014). We employed C.FVB-Tg(*Gnrhr-luc/EGFP)L8Mrln*/LmwJ transgenics ("Glowing Head"; GH) as a "tolerized" host (Day et al., 2014). These mice exhibit a tissue-restricted pattern of ffLUC/GFP expression that ostensibly generates peripheral tolerance to both ffLUC-expressing and GFP-expressing cells. We tested a 4T1-ffLUC-eGFP line in the context of NOD-SCID, wild-type (WT), and GH transgenics (Figure S1G). Similar to the 4T1-high line, 4T1-ffLUC-eGFP cells were rejected by WT hosts (Figure S1H). To our surprise, the same degree of tumor rejection was encountered in GH hosts (Figure S1H). When we assessed the number of GFP-specific (Figure S1I) or ffLUC-specific (Figure S1J) $CD8^+$ T cells through the use of class I tetramer assays, we observed that GH hosts generated comparable numbers of GFP- and ffLUC-specific cytotoxic T cells to those in WT mice following inoculation with the 4T1-ffLUC-eGFP line. Bioluminescent imaging as a correlate for metastatic outgrowth indicated lack of metastases in both WT and GH mice across organ sites (liver, lung, brain, and bone; Figure S1K). These data suggest that expression of GFP in extra-thymic tissues insufficiently tolerizes an animal to GFP, and the result is failure of metastases to emerge from a highly metastatic cell line.

Based on these and other data, we began to suspect that unimpeded progression of GFP^+ tumor cells would require a transgenic that is tolerized *centrally* to GFP, meaning one that expresses GFP in a significant proportion of thymic-antigen-presenting cells (Malhotra et al., 2016). We hypothesized that transgenic mice that express GFP in dendritic cells

would be centrally tolerized and thus could be leveraged for our purposes. To this end, we employed the *Cx3cr1-GFP;CCR2-RFP* model (referred to as "*Cx3cr1-GFP*") (Jung et al., 2000). We orthotopically transplanted 4T1-high cells into immune-compromised (NOD-SCID), immune-competent (WT), or GFP-tolerized (*Cx3cr1-GFP*) mice (Figure S1L). In the context of centrally tolerized *Cx3cr1-GFP* transgenics, 4T1-high cells now generated tumors with dynamics similar to those of the parental 4T1 line in WT mice (Figure S1M). Moreover, tumoral GFP was now maintained at levels comparable to those of 4T1-high cells within immune-compromised hosts (Figure S1N). GFP-specific $CD8^+$ T cells were not detected through the use of class I tetramer assays nor via ImmunoSpot in *Cx3cr1-GFP* mice (Figure S1O–P). The absence of an immunological response to GFP coincided with highly penetrant GFP^+ lung metastases in this strain, and these metastases were distinguished from host immune cells by CD45-negativity (Figure S1Q). Of note, the notion that centrally tolerized mice permitted progression and metastasis of GFP^+ cells was not BALB/c-specific; similar data were obtained when employing a centrally tolerized C57BL/6 strain (*Aire-GFP*) (Gardner et al., 2008) inoculated orthotopically with a syngeneic mammary tumor line (E0771) or subcutaneously with a lung cancer line (LLC1; data not shown) that expresses eGFP.

These data explicitly define a GFP-directed $CD8^+$ T cell response that suppresses primary tumor progression and exerts an even narrower bottleneck at the metastatic site. Equally as important, our work establishes design principles that are necessary to overcome this response—namely, employment of centrally tolerized transgenics that do not merely attenuate GFP-directed immune responses but eliminate them altogether (Figure S1R). We acknowledge that the drastic effect on tumor progression documented in these contexts might be relieved partially by utilizing alternative fluorescent proteins, tuning transgene expression level, injecting more or fewer cells, or changing the cell line(s) or mouse strain(s) employed. Nevertheless, we contend that, despite the nuanced adjustments to primary tumor progression these manipulations may achieve, the more overt phenotype at metastatic sites will remain. Focusing effort instead on development and adoption of centrally tolerized models should facilitate tumor transplantation studies of immune-related phenomena that are not skewed by profound, non-physiologic responses to fluorescent and/or luminescent neo-antigens.

Supplementary Material

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