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**Pardoll and Allison reply:**

In response to our commentary defining potential barriers to development of novel immunotherapy opportunities, Skipper and colleagues describe a unique cancer immunotherapy consortium developed by the Ludwig Institute for Cancer Research (LICR) and joined by the Cancer Research Institute (CRI). The LICR-CRI cancer vaccine collaborative (CVC) funds units in roughly 20 institutions. These units participate in a set of coordinated cancer vaccine clinical trials focused on vaccination with a limited number of tumor antigens identified at LICR and evaluates both the generation of antigen-specific immune responses as well as clinical outcomes. As director of both institutes, Dr. Old has marshaled their vast collective resources in a visionary manner to create a multi-institutional mini-Manhattan Project. CVC's funding base allows the organization to circumvent some of the regulatory and 'public-private partnership' barriers outlined in our commentary. However, the LICR-CRI CVC is arguably so unique, it represents the exception that proves the rule.

The projects being developed within the LICR-CRI CVC represent a small fraction of the tremendously promising opportunities provided by the past 10 years of accelerated molecular immunology and oncology research. If 10 such 'immunotherapy collaboratives' were similarly funded, with infrastructure to facilitate productive interactions among them and with the US FDA as well as the private sector, then a reasonable proportion of the crop could be harvested.

Where could such resources come from? Skipper and colleagues suggest that leading academic centers reallocate institutional resources to build translational infrastructures similar to that of the LICR-CRI CVC. Unfortunately, given the ever-increasing financial strains on the provision of health care within academic medical centers, it is unrealistic to expect resources of comparable scope to become available through individual institutions. Even the CVC's resources provide limited leverage to mobilize the immunologic agents currently languishing within biotechnology and pharmaceutical company portfolios. Congress now allocates the NCI $5 billion per year to mobilize the most effective anticancer effort possible. While still a tiny fraction of the current military budget, it is a resource that cannot be ignored and must be leveraged as effectively as possible. In addition, no serious therapeutic development can or should go on without the active participation of the FDA. While we applaud the unique structure and opportunities offered by the LICR-CRI CVC, and hope it will inspire the creation of analogous efforts, the key governmental institutions charged with protecting and promoting the health of Americans must be solidly on the playing field working with individual investigator groups at academic institutions and the corporate world alike to develop effective combinatorial therapies for human cancer therapy.

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# Active tissue factor in blood?

**To the editor:**

Following mechanical or chemical damage of the vessel or monocyte stimulation, tissue factor is exposed to the blood and binds plasma factor VIIa (FVIIa) forming the FVIIa–tissue factor enzyme complex. During the last several years, a number of studies have reported that physiologically active tissue factor is found circulating in blood of healthy individuals either as a component of blood cells and microparticles or as a soluble plasma protein[1]. Reports of the presence, source and activity of tissue factor in blood are controversial, with reported concentrations of physiologically active tissue factor varying from undetectable (<60 fM) in whole blood[2] to as high as 37 pM in the plasma of healthy individuals[3]. Blood or plasma activated with (sub)picomolar concentrations of functional tissue factor clots within several minutes (**Fig. 1**), suggesting that such concentrations of functional tissue factor cannot be present in blood or plasma *in vivo*.

We titrated tissue factor into fresh nonanticoagulated blood from healthy individuals in the presence of the corn trypsin inhibitor (CTI). CTI suppresses the contact pathway initiation of coagulation by inhibiting factor XIIa[4]. In the absence of exogenous tissue factor, CTI-blood kept at 37 °C with mixing does not clot for >1,200 s (**Fig. 1**). The addition of as little as 20 fM of tissue factor to blood treated with CTI resulted in clot formation in 1,000 s. Titrations of tissue factor resulted in shortening of the blood clotting time in a tissue factor concentration–dependent manner. The coagulation response observed at a concentration of tissue factor as low as 20 fM leads to the conclusion that this concentration of functional tissue factor must be well beyond that present in blood from healthy individuals. Over the past 9 years, we have performed >300 tissue factor–initiated whole blood clotting experiments using many donors, multiple phlebotomists and different CTI and tissue factor preparations. In virtually all of these experiments, the clotting time in the absence of added tissue factor was 20 min or greater (extending up to 40 min)[4,5].

The potential origins of discrepancies in the detection of active blood tissue factor are of interest. The most commonly used tissue factor activity assay evaluates factor Xa generation in the presence of FVIIa. In this assay, supraphysiologic concentrations of FVIIa are used, frequently exceeding those circulating *in vivo* (~0.1 nM) by two orders of magnitude[6]. At these FVIIa concentrations, the soluble form of tissue factor (an extracellular domain of tissue factor) can bind FVIIa and display limited proteolytic activity. At the physiologic FVIIa concentration, however, soluble tissue factor displays negligible activity[7] and is not likely to trigger blood coagulation; as a competitor, it would most likely inhibit coagulation.

In a recent study, the authors used physiologically irrelevant conditions to

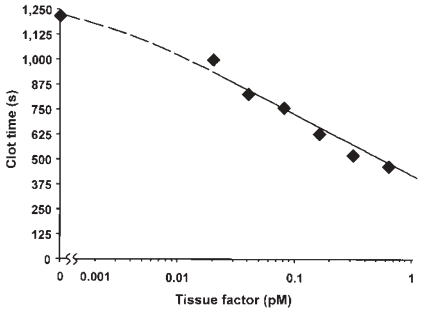


**Figure 1** Titration of relipidated tissue factor in contact pathway–inhibited whole blood. Varying concentrations of tissue factor were added to CTI-blood, and clotting times were evaluated visually.

assess plasma clotting activity of blood tissue factor[8]. The concentration of a soluble form of blood tissue factor was almost five orders of magnitude higher than that reported by the same authors in plasma. But even under these conditions, the authors detected only limited activity, which was substantially lower than that observed for full-length tissue factor; this limited level of activity is similar to that reported for the extracellular domain of tissue factor[7]. Additionally, the limited decrease in the clotting time reported by Bogdanov *et al.*[8] (from 230 to 150 s) may be caused by uncontrolled contact pathway activity (that is, by the surface of the tube). Conventional assays with clotting times of >100 s without use of a contact pathway inhibitor are questionable. The hypothesis that soluble blood tissue factor is required to promote thrombus growth is more speculative than supported by experimental data. Although Bogdanov *et al.*[8] observed the accumulation of soluble tissue factor in the growing thrombus, no data suggesting the activity of this tissue factor were provided; potentially, soluble tissue factor may act as an inhibitor of coagulation by binding FVIIa into an inactive complex.

The addition of 5 pM active tissue factor to blood provides a clotting time of ~5 min (current study and previously published[5]), that is, similar to that observed in 'Simplate' bleeding assays[9]. The observation that active tissue factor in blood of healthy individuals does not exceed a concentration of a few femtomolar suggests that if clotting were to require blood-borne tissue factor, the concentration of this protein at the site of injury would have to increase by at least three orders of magnitude above that normally present. As a consequence, over the time required to accumulate enough blood tissue factor activity at the site of the injury, a life-threatening blood loss (>3 L) would occur even in the case of minor damage to the vasculature.

In conclusion, we believe that there are no reliable data suggesting the presence of significant quantities of functional tissue factor in the blood of healthy individuals. Blood donors were recruited according to a protocol approved by the University of Vermont Human Studies Committee, and their consent was obtained.

COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

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## The authors reply:

We do not question the validity of the measurements presented by Butenas and Mann, particularly because it had been previously shown that tissue factor antibodies did not prolong blood clotting times[1]. Nor do we dispute their statement that alternatively spliced tissue factor, which does circulate[2], has minimal activity, although the tested preparations were derived from *Escherichia coli*, perhaps thereby underestimating its activity. Moreover, this protein is found in all thrombi, thus supporting its further study.

Whole-blood clotting times are performed using nonflow conditions and therefore do not address the participation of blood-borne tissue factor in thrombogenesis, for which there is considerable evidence, apparently ignored by Butenas and Mann. Giesen *et al.*[3] showed that *ex vivo* deposition of fibrin-containing thrombi on a collagen surface was virtually eliminated by a monoclonal antibody to tissue factor. Inasmuch as these experiments used no tissues other than blood, one must conclude that blood contributed the tissue factor. An *in vivo* model developed by Himber *et al.*[4], in which thrombus growth within rabbit jugular veins or within a silastic jugular-jugular shunt was evaluated by radiolabeled fibrin accretion, rose linearly with time until a monoclonal antibody to rabbit tissue factor was perfused, after which there was essentially no thrombus growth. Further, a mouse transgenic model supported the role of circulating tissue factor in thrombogenesis[5].

A fundamental difference between the cited experiments and the current data of Butenas and Mann is that the latter measured *in vitro* blood coagulation under nonflow conditions whereas the former studied thrombus formation in flowing blood. It is abundantly clear that shear forces caused by laminar flow are involved in thrombogenesis[6]. A potentially critical observation emphasizing the role of shear force is that fresh platelets do not stain for tissue factor; however, following perfusion with tissue factor–positive cells under physiologically relevant flow conditions, platelet aggregates were markedly positive for tissue factor[7].

In summary, we do not dispute the results presented by Butenas and Mann; we do, however, seriously question the applicability of data derived from static assays to thrombogenesis, as well as the underlying rationale for this extrapolation.

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