RESEARCH ARTICLE

Anticoagulant properties of the anti-inflammatory cytokine IL-10 in a factor Xa-activated human monocyte model

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ABSTRACT. ***Background:*** **Monocytes and factor Xa (FXa) are procoagulant agents implicated in the physiopathological processes of atherosclerosis and thrombosis.*Objective:* we evaluated the anticoagulant effect of the anti-inflammatory cytokine IL-10 on an FXa-activated human monocyte (Hu-monocyte) procoagulant phenotype.*Methods:* Hu-monocytes were purified by elutriation and activated by FXa. The effect of IL-10 was assessed by means of a 2 h pre-incubation step with recombined human IL-10 (0.5 and 1 ng/mL). Real-time RT-PCR and Western blotting were used to evaluate the effect of IL-10 on tissue factor (TF) mRNA and protein synthesis. A thrombin generation (TG) assay was used as a functional test to assess the effect of IL-10 on TF-dependent TG.** ***Results:*** **we showed that IL-10 inhibited both TF mRNA and TF protein expression in a dose-dependant manner. We showed, as a functional consequence, that IL-10 inhibited Hu-monocyte-triggered TG and that this inhibition was concentration-dependant, and significant for all TG phases. The rate index of the propagation phase (rate index) was the most sensitive parameter while the endpoint of TG decay (S-tail) and the endogenous thrombin potential (ETP) were the least sensitive (inhibition of 80, 40 and 30% respectively). The IL-10 pattern of TG inhibition was similar to TF-Ab-induced inhibition: IC_{50} were not reached by ETP and S-tail, and the lowest IC_{50} values were reached by the rate index (0.61 ± 0.12 ng/mL and 1.87 ± 0.35 µg/mL respectively).*Conclusion:* the anticoagulant effect of the anti-inflammatory cytokine IL-10 in an FXa-activated Hu-monocyte model is an additional illustration of the cross-talk between inflammation and coagulation, opening new approaches in the field of arteriosclerosis and thrombosis.**

Key words: factor Xa, human monocytes, interleukin-10, thrombin generation, tissue factor

Inflammation and coagulation are closely linked in a variety of pathological processes such those related to atherosclerosis and thrombosis [1-3]. In these disorders, monocytes are recognized as playing a key inflammatory role through their production of pro-inflammatory cytokines, eicosanoides and reactive oxygen species, and complement activation [4-6]. Inflammation leads frequently to a coagulation disturbance, and monocytes are key promoters of inflammation-induced coagulation through their ability, upon activation, to express membrane tissue factor (TF) [7, 8], and to release TF-rich monocyte-derived microparticles [9, 10]. TF is an integral membrane protein that, in contact with coagulation factor VII/VIIa, initiates the extrinsic coagulation cascade by activating factor X to factor Xa (FXa) that then triggers thrombin generation (TG) and thrombus formation [11]. FXa also promotes coagulation through the induction of monocyte TF expression and then monocyte procoagulation [12]. FXa forms a keystone in the crosstalk between coagulation and inflammation since it triggers, *via* protease-activated receptor (PARs) activation, a broad range of signalling pathways leading to inflammatory response [2, 13]. Activation of PARs leads to the release of the anti-inflammatory interleukin (IL)-10 [14]. IL-10 is a multifunctional cytokine that influences inflammation by inhibiting a broad spectrum of activated monocyte functions including inhibition of:

– pro-inflammatory cytokine expression [15, 16],
– reactive oxygen intermediate release [17],
– inter-cellular adhesion molecule expression [18, 19],
– apoptosis induction [20, 21].

A potential IL-10 anticoagulant effect was also reported using LPS models [9, 22]. However, it is not known whether IL-10 can counteract the FXa-induced monocyte procoagulant phenotype. The present study was designed to evaluate the effect of IL-10 on FXa-activated Hu-monocyte TF mRNA and protein expression, and on TG triggered by FXa-activated Hu-monocytes.

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METHODS

Platelet-poor plasma preparation

Venous blood samples were obtained from five healthy volunteers (mean age 27 ± 4 years), informed consent having been obtained from each participant. Volunteers were laboratory staff members and had been medication-free for the previous two weeks. Blood was withdrawn by antecubital venipuncture into Monovette® tubes (0.106 M citrate, BD, Franklin Lakes, NJ, USA) and centrifuged for 10 min at 190 *g*, followed by 10 min at 1,750 *g*, and 30 min at 13,000 *g*. Platelet-poor plasma (PPP) supernatants were pooled and stored at -80°C. They were thawed for 5 min at 37°C, immediately before use.

Monocyte purification

Cytapheresis material was obtained from four healthy volunteers admitted for platelet donation at the blood transfusion unit of CHU Robert Debré (Paris, France). Informed consent was obtained from all participants. Human monocytes (Hu-monocytes) were purified from cytapheresis residues by elutriation, as previously described [23]. Monocyte purity was evaluated by CD14 staining of isolated cells (>95% CD14-positive), and cell viability (>98%) was assessed by the trypan blue exclusion principle.

Monocyte activation

Purified Hu-monocytes were washed in RPMI-1640 medium (Invitrogen, Cergy Pontoise Cedex, France), adjusted to 5.0×10^6 cells/mL in the same medium, and then activated by FXa (Stachrom heparin kit®, Diagnostica Stago, France) at 37°C in a 5% CO_2 humidified atmosphere. FXa was used at a concentration of 1 U/mL, and Hu-monocytes were incubated for 3 h (real-time RT-PCR) or 5 h (Western blotting and TG test). Supernatants were removed by centrifugation for 5 min at 400 *g*; the pellets were washed with PBS (BioMérieux, France) and were either resuspended in 150 μL PBS (TG test), or stored as dry pellets at -80°C (real-time RT-PCR and Western blotting). The level of endotoxin in the FXa solution was determined by ELISA, as per manufacturer's specifications (Limulus Amebocyte Lysate (LAL) Kinetic-QCL®; Lonza, France). FXa solution was found to be endotoxin-free.

IL-10 treatment of monocytes

Evaluation of the effect of IL-10 was assessed through a pre-incubation step with recombined human IL-10 (R&D Systems, France) prior to FXa activation. Monocytes, adjusted to 5.0×10^6 cells/mL in RPMI, were pre-incubated for 2 h with IL-10 (0.5 and 1 ng/mL) at 37°C in a 5% CO_2 humidified atmosphere. There was no washing step prior to FXa activation.

RNA extraction and real-time RT-PCR

Total RNA was extracted from Hu-monocytes (5.0×10^6 cells) using an RNeasy Mini-Kit™ (Qiagen, France). Total cellular RNA (1 μg) was used for cDNA synthesis using the iScript cDNA synthesis kit (Biorad, France). The cDNAs were then subjected to real-time RT-PCR analysis with Sybr Green PCR core reagents, as previously described [9]. Forward and reverse oligonucleotide primers were as follows:

- β2-microglobulin:
  - forward, 5′-CCC CCA CTG AAA AAG ATG AG-3′
  - reverse, 5′-TCA TCC AAT CCA AAT GCG GC-3′
- TF:
  - forward, 5′-CCG ACG AGA TTG TGA AGG ATG T-3′
  - reverse, 5′-AGA GGC TCC CCA GCA GAA C-3′

Data were analyzed with the 7000 System SDS software. The transcript for the constitutive gene product β2-microglobulin was used for data normalization.

Protein extraction and Western blotting

Activated Hu-monocytes (dried pellet of 5.0×10^6 cells) were lysed in RIPA buffer (1% igepal, 0.5% sodium deoxycholate, 0.1%SDS), in the presence of protease inhibitors (Complete Protease Inhibitor Cocktail ®, Roche, France). Protein concentrations were determined using a Bradford kit (Biorad, France). Protein samples (10 μg) were subjected to 10% SDS–PAGE followed by a transfer to a PDVF membrane (Immune-Blot™ PVDF; Biorad, France), an incubation overnight with mouse anti-human FT antibody (4509; American Diagnostica, France), then 1 h with horseradish peroxidise (HRP)-coupled goat-anti-mouse secondary antibody (IM0817; Beckman Coulter, France), and subsequent chemiluminescent detection using an ECL Plus™ Western blotting detection kit (Amersham, France). The TF signal was identified using a pre-stained, molecular mass protein ladder (Euromedex, France).

Fluorogenic measurement of thrombin generation

The TG test was performed as previously described [24]. PPP was systematically used, and was supplemented with aprotinin (Sigma, France) at 200 kallikrein inhibitory units (KUI)/mL. Il-10-pre-treated FXa-activated Hu-monocytes (20 μL; 0.4×10^6 cells) were added to 80 μL of PPP. The fluorogenic substrate, Z-Gly-Gly-Arg-AMC (Bachem, Switzerland) was added, and fluorometric determination of TG was performed using a Fluoroskan® Ascent plate reader (ThermoLabsystems, Finland). Thrombinoscope™ software (Synapse BV, The Netherlands) was used to calculate TG. Six parameters were analyzed:

(a) time of TG initiation (lag-time, min),
(b) time to reach thrombin peak [tt-peak, min],
(c) thrombin peak [peak, nM],
(d) endogenous thrombin potential [ETP, nM.min],
(e) rate index of propagation phase, calculated by the formula peak/(time to peak – lag-time) [rate index, nM/min],
(f) the endpoint of the decay of thrombin formation [S-tail, min].

The inhibitory effect of anti-TF antibodies TF-Ab (American Diagnostica, France) was evaluated by incubating FXa-activated Hu-monocytes, free of IL-10, with increasing TF-Ab concentrations (1, 5, and 10 μg/mL) for 30 min prior to TG testing.

Statistical analysis

Statistical analysis was performed on SPSS v. 17.0 software (SPSS, Chicago, IL, USA). Data were expressed

as mean ± SD and Mann-Whitney was used for statistical comparisons. $p < 0.05$ was considered as statistically significant.

RESULTS

Effect of IL-10 on Hu-monocyte TF expression

RT-PCR and Western blot results showed that IL-10-pre-incubated Hu-monocytes exhibited a decrease in TF mRNA and TF protein expression compared to Hu-monocytes activated by FXa and not pre-treated with IL-10 (*figure 1A-B*). IL-10 inhibition of both TF mRNA and TF protein expression was observed in a dose-dependant manner. Two negative controls were used: monocytes free of IL-10/FXa and monocytes with IL-10 alone.

Effect of IL-10 on the Hu-monocyte thrombin generation phenotype

The effect of IL-10 on TG triggered by FXa-activated Hu-monocytes was tested at 0.5 and 1.0 ng/mL. We observed an inhibition of TG at both concentrations *versus* no IL-10 pre-treatment (*figure* 2A). Inhibition was concentration-dependant, and was significant for all TG parameters except ETP at 0.5 ng/mL (*figure 2B-G*). The most sensitive parameter to IL-10 inhibition was the rate index (inhibition of 80%); the sensitivity was intermediate for lag-time, tt-peak, and peak (inhibition from 55 to 65%), and lower for S-tail (inhibition of 40%) and ETP (inhibition of 30%). Values for TG parameters at baseline (no IL-10), C1 (IL-10 0.5 ng/mL) and C2 (1.0 ng/mL) were:

– for lag-time:
  - 3.3 ± 0.6 min,
  - 4.3 ± 1.1 min,
  - 7.2 ± 1.3 min,
– for tt-peak:
  - 7.1 ± 1.4 min,
  - 9.4 ± 2.4 min,
  - 18.9 ± 3.9 min,
– for peak:
  - 202 ± 18 nM,
  - 169 ± 37 nM,
  - 86 ± 26 nM,
– for ETP:
  - 2.262 ± 122 nM.min,
  - 2.131 ± 119 nM.min,
  - 1.620 ± 322 nM.min,
– for rate index:
  - 57 ± 12 nM/min,
  - 38 ± 21 nM/min,
  - 12 ± 6 nM/min,
– for S-tail:
  - 41 ± 3 min,
  - 48.3 ± 10.6 min,
  - 69.0 ± 10.9 min.

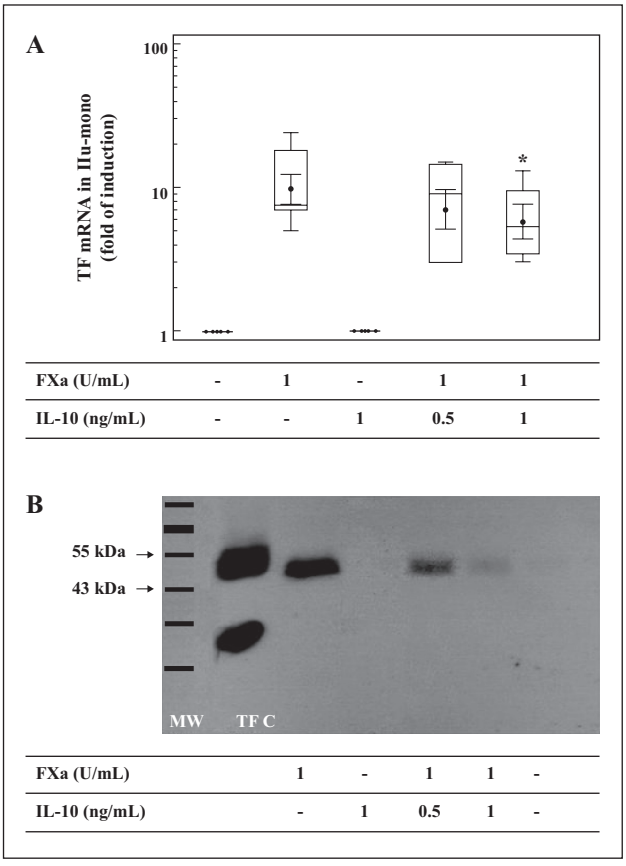


Figure 1
IL-10 inhibition of FXa-induced TF mRNA and protein expression. **(A)** Fold induction of TF mRNA quantified using real time RT-PCR; Box plots were obtained from four duplicate experiments and p evaluated by the Mann–Whitney test. * $p<0.05$ when compared to FXa activation. **(B)** TF protein synthesis evaluated by Western blot. TF positive control (TF C) was a mix of Thromborel® (45 kD, glycosylated TF) and Innovin® (34 kD, recombinant non-glycosylated TF). *MW*: molecular weight marker.

Comparison between IL-10 and TF-Ab in TG modulation

In this section, the inhibitory effect of IL-10 on TG is compared to its effect on TF-Ab. For this, three concentrations of TF-Ab were used. A concentration-dependant inhibition of TG (*figure 3*) was observed. Time parameter extend were of 4, 3.5 and 1.5 times for lag-time, tt-peak and S-tail respectively and the maximal percentages of TG inhibition were 75% for peak and rate index, and only 35% for ETP. The TF-Ab IC_{50} were then determined and compared to those of IL-10 (*table 1*). For peak, ETP and rate index, IC_{50} was defined as the IL-10/TF-Ab concentration that allowed a decrease of 50%. For time parameters, *i.e.* lag-time, tt-peak and S-tail, IC_{50} was defined as the IL-10/TF-Ab concentration that doubled the time. IC50 could not be calculated for ETP and S-tail for concentrations used (with either for IL-10 or TF-Ab) (*table 1*). Conversely, it could be calculated for any other parameter. The lowest IC_{50} values for both IL-10 and TF-Ab were reached by rate index (0.61 ± 0.12 ng/mL for IL-10, and 1.87 ± 0.35 μg/mL for TF-Ab).

DISCUSSION

In a model of *in vitro* FXa-activated Hu-monocytes, we showed, for the first time, that IL-10 is able to circumvent TF mRNA induction and TF protein expression. We demonstrated, as a functional consequence, that IL-10 inhibited FXa-activated Hu-monocyte-triggered TG. Our results support the complexity of the bimodal crosslink

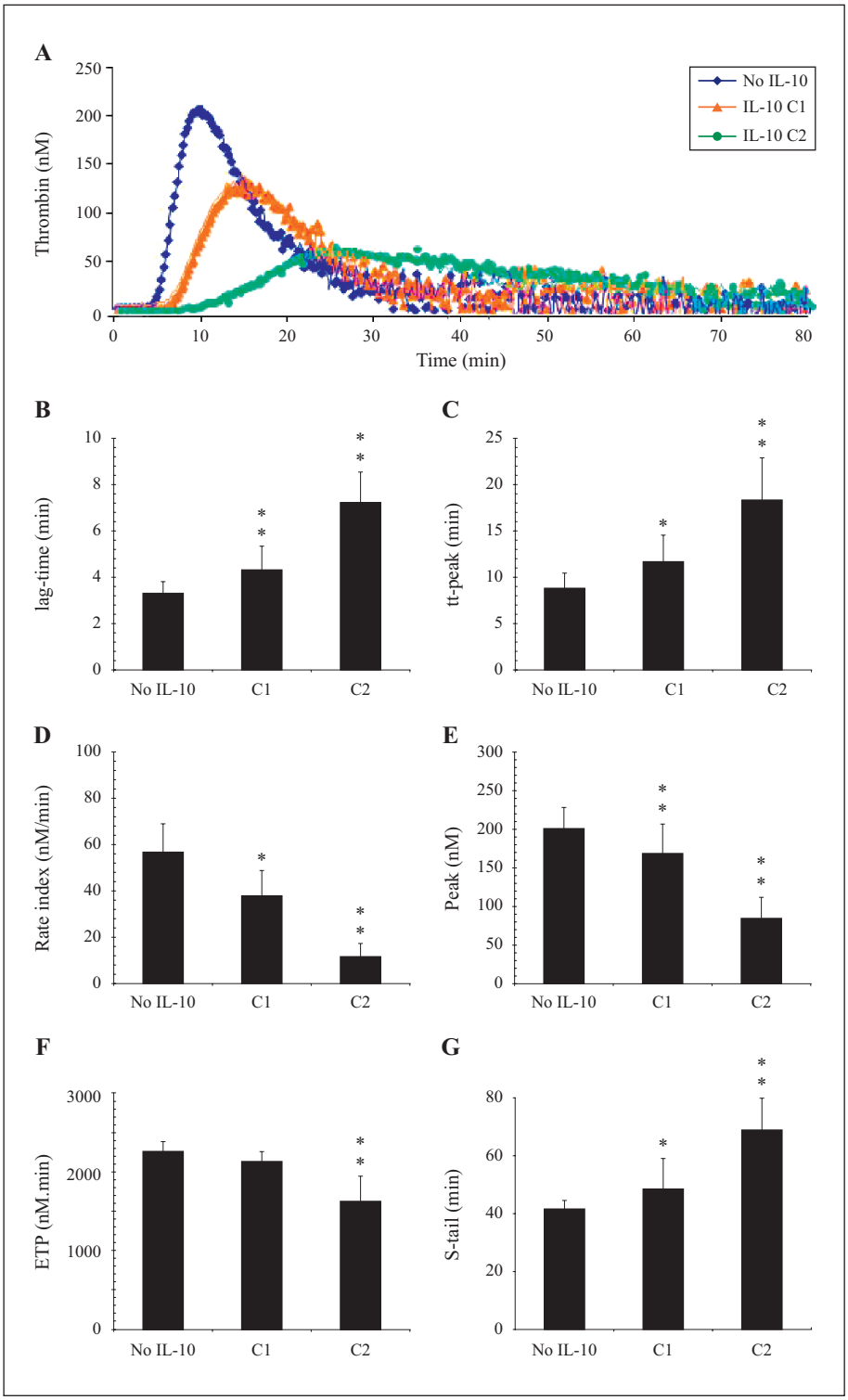
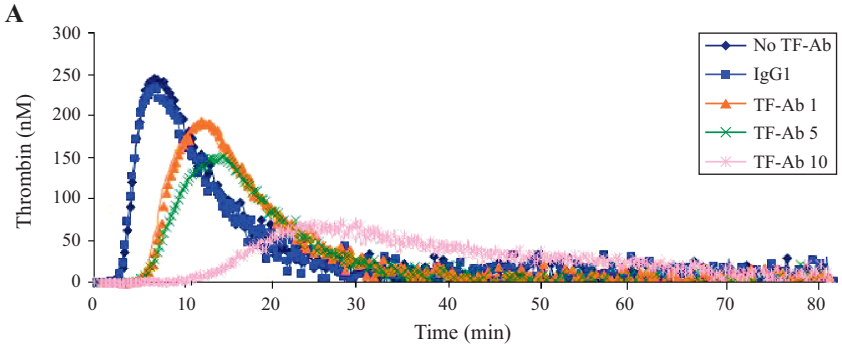


Figure 2

Effect of IL-10 the Hu-monocyte thrombin generation (TG) phenotype. IL-10 was tested at C1 (0.5 ng/mL) and C2 (1.0 ng/mL). Thrombogram **(A)** is a representative thrombogram from four triplicate experiments. Histograms (b-g) represent means ± SD of six TG parameters: **(B)** time of TG initiation (lag-time), **(C)** time to reach thrombin peak [tt-peak], **(D)** thrombin peak [peak], **(E)** endogenous thrombin potential [ETP], **(F)** rate index of propagation phase, [rate index], and **(G)** the endpoint of TG decay [S-tail]. * $p<0.05$ *versus* absence of IL-10 pre-treatment. * $p<0.05$ *versus* both absence of IL-10 pre-treatment and previous IL-10 concentration.

between coagulation and inflammation [1-3]. Indeed, it has been well demonstrated that pro-inflammatory cytokines promote procoagulation [7, 25] and that inversely procoagulant factors promote the inflammatory response [13, 26, 27]. It has also been reported that anticoagulant molecules might transmit anti-inflammatory responses [28, 29]. Conversely, we have shown that the anti-inflammatory cytokine IL-10 could act as an anticoagulant molecule. Our results are in line with previous data showing that IL-10 inhibits LPS-induced coagulation [9, 22]. In our model, we chose to activate Hu-monocytes by FXa, instead of the non-physiological trigger LPS. FXa, initially only considered as a passive bystander in blood coagulation, has been recently suggested to be a pivotal keystone in the crosstalk between coagulation and inflammation [2, 3]. FXa is described as a crucial factor in the



B

	TF-Ab (μg/mL)			
	0	**1**	**5**	**10**
lag-gtime (min)	**2.8 ± 0.9**	**4.5 ± 0.8[1]**	**5.6 ± 0.7[1,2]**	**11.1 ± 0.3[1,2]**
tt-peak	**5.6 ± 1.8**	**9.5 ± 2.8[1]**	**13.1 ± 1.4[1,2]**	**19.9 ± 2.2[1,2]**
rate index (nM.min^{-1})	**103 ± 38**	**55 ± 25[1]**	**36 ± 22[1,2]**	**27 ± 8[1,2]**
peak (nM)	**270 ± 25**	**191 ± 26[1]**	**158 ± 43[1,2]**	**70 ± 24[1,2]**
ETP (nM.min)	**2579 ± 78**	**2443 ± 121[1]**	**2086 ± 91[1]**	**1600 ± 122[1,2]**
S-tail (min)	**40 ± 2**	**47 ± 2[1]**	**48 ± 1[1]**	**66 ± 4[1,2]**

1 : p<0.05 when compared to the absence of TF-Ab
2 : p<0.05 when compared to previous TF-Ab concentration

Figure 3
Effect of increasing concentrations of TF-Ab on the Hu-monocyte thrombin generation (TG) phenotype. TF-Ab concentrations are C1 (1 μg/mL), C2 (5 μg/mL) and C3 (10 μg/mL). **(A)** A representative thrombogram **(B)** Data obtained from four triplicate experiments (means ± SD).

Table 1
Comparison between IL-10 and TF-Ab in TG modulation.

TG parameters	**IC_{50} values**	
	IL-10 (ng/mL)	**TF-Ab (μg/mL)**
lag-time	0.89 ± 0.12	5.13 ± 1.36
tt-peak	0.89 ± 0.02	4.97 ± 1.73
Rate index	0.61 ± 0.12	1.87 ± 0.35
Peak	0.94 ± 0.05	6.43 ± 0.04
ETP	ND (30%)	ND (35%)
S-tail	ND (40%)	ND (40%)

Results from four triplicate experiments
ND not determined
(%) maximal percentage of inhibition

physio-pathological processes related to atherosclerotic and thrombotic disorders. Indeed, FXa is implicated in a broad range of intracellular signalling leading to induction of cell TF expression, cell-triggered TG, and in the promotion of coagulation [12]. FXa is also an inflammatory promoter by its ability to induce production of pro-inflammatory cytokines [2, 13], which in turn promote inflammation-driven coagulation [7, 25]. FXa acts by activating PARs. These receptors are largely expressed and localized in the vasculature on endothelial cells, monocytes and platelets, and their activation advances atherosclerosis and related thrombosis [2].

To evaluate the effect of IL-10, we used the thrombin generation assay, instead of current coagulation assays. Indeed, classical chronometric assays evaluate the very initial phase of coagulation, since 10 nM of thrombin are sufficient for clotting. Conversely, TG provides information on the whole process of coagulation in plasma [30, 31]. Here, we show that IL-10 inhibits all phases of TG in a dose-dependent manner. In this model, the most sensitive TG parameter to IL-10 inhibition was the rate index of the propagation phase, the less sensitive parameters being ETP and S-tail. As we hypothesized that inhibition by IL-10 was linked to FT down-regulation, we compared IL-10 to TF-Ab using the TG phenotype. We observed similar patterns of inhibition as those demonstrated by calculation of IC_{50}. Accordingly, IL-10 can be considered to be an indirect anti-Xa molecule. In line with this conclusion, we previously reported the inhibitory effect of fondaparinux in the same model of FXa-activated Hu-monocytes TG. The pattern of inhibition induced by fondaparinux indicates the rate index as having the highest, and ETP the lowest sensitivity [12].

Here we have demonstrated the anticoagulant effect of the anti-inflammatory cytokine IL-10 in a FXa-activated Hu-monocyte model, *via* a modulation of TF expression. This is an additional illustration of the cross-talk between inflammation and coagulation, opening new approaches in the field of arteriosclerosis and thrombosis.

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