Tissue Factor Induction in Human Monocytes

Two Distinct Mechanisms Displayed by Different Alloantigen-responsive T Cell Clones

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Abstract

One component of the cellular immune response to antigens is the expression of procoagulant activity (PCA) by monocytes and macrophages. Induction of human monocyte PCA in response to alloantigenic stimulation requires the collaboration of HLA-DR-responsive T cells. In mixed lymphocyte cultures (MLCs), the induction of monocyte tissue factor appears to be mediated exclusively by a T cell-derived lymphokine. We have used a soft agar cloning method to generate alloantigen-responsive T cell clones from MLCs between irradiated Daudi lymphoblastoid cells and human peripheral blood mononuclear cells. Developing clones were screened for the ability to induce PCA in fresh autologous monocytes in response to Daudi stimulator cells. PCA induction was observed with some, but not all, proliferating T cell clones and two modes of induction were apparent. Some T cell clones mediated PCA induction exclusively by lymphokine production, whereas other clones delivered induction signals by direct cellular collaboration with the monocyte effector cells. These two inductive pathways were represented in distinct, non-inclusive functional subsets of T cell clones. Constitutive production of soluble inducer signals was not observed in T inducer clones. The magnitude of the monocyte PCA response increased in response to an increase in the allogeneic stimulator/T clone responder ratio, and third-party allogeneic cells were unable to elicit the PCA-inducing lymphokine signals from T inducer clones. Both modes of induction were shown to generate tissue factor protein activity in monocytes. Collectively, these results suggest that PCA induction can be initiated in response to alloantigens through collaboration with certain OKT3$^+$, OKT4$^+$, OKT8$^-$, OKM1$^-$ T inducer clones, and that induction can be mediated by at least two different functional subsets of human T cells. Stimulation with the appropriate alloantigen may elicit both lymphokine and T cell–contact pathways of induction of tissue factor in human monocytes.

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Introduction

Initiation of the extrinsic coagulation protease cascade is a concomitant event in many immune T cell-driven monocyte–macrophage effector cell responses (1). Expression of the tissue factor protein, the initiation cofactor for this effector function, can be induced collaboratively on monocytes and macrophages by a variety of antigenic and nonantigenic stimuli including bacterial endotoxins (2), plant lectins (3), small soluble immune complexes (4), soluble protein antigens (5), viral antigens (6), and cellular alloantigens (7–9). The monocyte tissue factor response initiates the extrinsic coagulation protease cascade by binding factor VII (10), and as such has been shown to be an important effector response in the immunopathogenesis of delayed-type hypersensitivity (11), viral infections (6), disseminated intravascular coagulation (12), and perhaps in allogeneic organ graft rejection (13). T lymphocytes have been shown to collaboratively initiate or amplify monocyte procoagulant activity (PCA)[1] responses to the eliciting stimuli. Although some agonists such as endotoxins, lectins, and immune complexes are able to induce tissue factor directly in appropriately responsive monocytes and macrophages (2–4), in the cases of immunologically specific stimuli T cell collaboration appears to be requisite rather than alternative for monocyte PCA expression (5, 14–17).

The monocyte PCA response in human mixed lymphocyte cultures (MLCs) has a rigorous requirement for T cell collaboration and, in response to Daudi lymphoblastoid cells, is apparently specific for HLA-DR (7). Induction of PCA in this system has appeared to be mediated solely through the production of a soluble T cell product, i.e., a lymphokine (16). Direct T cell–monocyte contact-mediated induction was not apparent when utilizing mixed T cell populations in which intercellular regulatory circuits among T cell subsets would be intact. We have recently generated and selected alloantigen-responsive, proliferating human T inducer clones, and we have characterized the ability of these clones to induce monocyte PCA in response to HLA-DR stimulation. We observe that both (*a*) cell-independent soluble lymphokine-mediated and (*b*) cell-associated tissue factor induction pathways are represented at the level of the T inducer clone. These two distinct collaborative pathways can be mediated by distinct subsets of T cell clones of the OKT3$^+$, OKT4$^+$, OKT8$^-$, OKM1$^-$ surface phenotype. The description of the T cell-associated pathway represents a new

1. *Abbreviations used in this paper:* FBS, fetal bovine serum; Il-2, interleukin 2; MLC, mixed lymphocyte culture; PBMC, peripheral blood mononuclear cell; PCA, procoagulant activity.

facet of T cell–monocyte collaboration in PCA responses to alloantigens.

Methods

Cell preparations. Human peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood of volunteer donors by buoyant density centrifugation on low endotoxin Ficoll-Hypaque (18). Monocytes were isolated from PBMCs on the basis of their receptor-mediated attachment to human plasma fibronectin on gelatin-coated petri dishes (19, 20). Daudi B lymphoblastoid cells were maintained in suspension culture in 75-cm^2 flasks (Costar, Cambridge, MA). Cells were prepared in low endotoxin RPMI 1640 (M. A. Bioproducts, Walkersville, MD) supplemented with 25 mM Hepes and 50 μg/ml of gentamycin. Complete RPMI 1640 was further supplemented with heat-inactivated, low-endotoxin fetal bovine sera (FBS) (K. C. Biological, Lenexa, KS). All cell culture media, FBS, and chemical reagents were screened for endotoxin contamination by the *Limulus* ambocyte lysate assay (E-toxate, Sigma Chemical Co., St. Louis, MO) and were found to be negative at the level of 0.01 ng/ml. Monocyte-enriched populations were >85% nonspecific esterase-positive (21) and >95% viable. Residual lymphocyte contamination in the monocyte-enriched populations was determined to be <5% by Giemsa staining.

Generation and culture of alloreactive T cell clones. Fresh PBMCs were stimulated with Daudi B lymphoblastoid cells which had been subjected to 8,000 rads of gamma-irradiation (Gammacell 40, Atomic Energy of Canada Ltd., Canada, Ontario) at a stimulator to responder ratio of 2:1 in complete RPMI 1640. Daudi cells did not proliferate after exposure to this amount of irradiation, and they did not possess constitutive PCA. After 48 h of MLC, lymphoblasts were recovered by Ficoll-Hypaque buoyant density centrifugation. Cloning was performed in a modified two-layer soft agar system based on the method of Rozenszajn et al. (22). The lower agar layer in each 35 × 10-mm dish (Costar) consisted of 2.5 ml of RPMI 1640 supplemented with 20% FBS, 0.0125 ml/ml phytohemagglutinin (purified grade, Wellcome Research Laboratories, Beckenham, UK), and 0.5% agar (Seaplaque agarose, FMC Corp., Rockland, ME). The upper layer contained 7.5×10^5 lymphoblasts in 0.85 ml of RPMI 1640 supplemented with 20% FBS, 20% bulk 48-h MLC supernatant, 4.5 U ml^{-1} of crude interleukin 2 (IL-2), and 0.33% agar. Crude IL-2 was produced by stimulating fresh human PBMC with phytohemagglutinin and phorbol myristate acetate (Sigma Chemical Co.) in the presence of irradiated allogeneic cells as described by Meuer et al. (23). Crude IL-2 activity was measured by uptake of [^{3}H]thymidine by murine HT-2 cells (24), and units of activity were based on an in-house rat IL-2 standard preparation (25) (a generous gift from Dr. Amnon Altman, Scripps Clinic and Research Foundation). The soft agar cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2, 95% air for 5 d. During this time 41±17 colonies developed per 10^5 cells plated, representing a cloning efficiency of 0.02–0.06% of the responder cell population. Each colony was composed of a cluster of 50–100 cells and were sparsely scattered through the agar. This cloning efficiency is consistent with previously published results (26) for soft agar cloning of alloreactive human T lymphocytes. Strict evidence for the clonal nature of each developing colony must come from the determination of a single HLA-DR recognition specificity. Efforts to that end are currently underway.

Individual colonies were removed from the upper soft agar gels under direct microscopic observation and transferred into U-bottomed microtiter plate (Costar) wells containing 2.5×10^4 irradiated Daudi stimulator cells and 2.5×10^4 irradiated autologous PBMCs (as feeder cells) in 0.1 ml of RPMI 1640 supplemented with 40% FBS and 3 U/ml of crude IL-2. Developing cultures were fed complete RPMI 1640 supplemented with 3 U/ml of crude IL-2 after 7 d. Cultures were split when they reached 10^5 viable cells per well and were restimulated at that time with 5×10^4 irradiated Daudi cells in complete RPMI 1640 with 3 U/ml of IL-2. ~1 wk later, growing cultures in two U-bottomed wells from each individual clone were combined and transferred into one well of a 24-well cluster plate (Costar) along with 1 ml of irradiated Daudi cells (5×10^5) and IL-2. Developing T cell clones were subsequently propagated in tissue culture flasks (Falcon, Becton-Dickinson Labware, Oxnard, CA) at a concentration of 5×10^5 cells/ml in complete RPMI 1640. Clones were fed fresh medium containing 1.5 U/ml of crude IL-2 every 4–5 d and were restimulated with irradiated Daudi cells (stimulator to responder ratio of 2:1) every 2–3 wk. Proliferation of the T cell clones was found to be dependent both on periodic restimulation by Daudi cells and on the addition of exogenous IL-2. The addition of either third party stimulator cells or medium without IL-2 to rested clones resulted in the arrest of proliferation within 3 d as determined by [^{3}H]thymidine uptake. Surface marker phenotypes of developing clones were determined by indirect immunofluorescence using monoclonal OKT3, OKT4, OKT8, and OKM1 followed by fluorescein isothiocyanate-goat anti-mouse IgG antibodies (Ortho Diagnostic Systems, Inc., Raritan, NJ) according to the manufacturer's protocol.

Screening of T cell clones for PCA inducer activity. T cell clones were screened at 10–14 d poststimulation for PCA inducer activity in two-stage experiments. In stage I, clones (2×10^5) were stimulated with various numbers of irradiated Daudi cells at a final concentration of 3×10^6 cells/ml for 18 h in complete RPMI 1640. The supernatants were recovered after centrifugation, and the pelleted cells were resuspended in the original volume of fresh medium. In stage II, 150 μl of the stimulated cells or corresponding supernatants was added to 5×10^4 autologous monocytes in 50 μl of complete RPMI 1640. After an additional 6 h of culture, the cells were recovered by centrifugation at 450 *g*, stored at −70°C, and later assayed for total cellular PCA. Unstimulated control cultures included monocytes cultivated for 6 h with complete RPMI 1640, irradiated Daudi cells, or the corresponding Daudi cell culture supernatants. All cultures were carried out in duplicate.

Assay for total cellular PCA. Cell pellets were solubilized at 37°C in 110 μl of Hepes-saline (pH 7.0) containing 15 mM β-octyl glucopyranoside (Calbiochem-Behring Corp., La Jolla, CA) as described previously (27). The duplicate reaction mixtures were diluted after 10 min with 140 μl of Hepes-saline, mixed by vortexing, and placed on ice. Samples (50 μl) were assayed in duplicate for PCA in a one-stage clotting assay (28) with 50 μl of citrated normal human platelet-poor plasma or factor-deficient plasma and 50 μl of 25 mM $CaCl_2$. Clotting times, measured from the point of addition of plasma to the point of formation of visible clots, were determined in duplicate tubes during continuous rocking in a 37°C water bath. Mean clotting times from individual reaction mixtures were converted to milliunits of PCA by reference to a rabbit brain thromboplastin standard (Difco Laboratories, Detroit, MI). Serial dilutions of the standard were used to produce a log–log plot, and a value of 10^5 mU/ml was assigned to thromboplastin at 37.5 mg of dry weight per milliliter (28). For further comparison, 10^3 mU/ml corresponded to a clotting time of 50 (48–53) s. Total cellular PCA is reported as milliunits per 10^5 monocytes and represents the mean±standard error of the mean in duplicate reaction mixtures. Precision of the array is substantiated by a coefficient of variation of 8%. The molecular specificity of the PCA was analyzed using hereditary factor IX-, VII-, and X-deficient plasmas that have been depleted of residual specific coagulation protein by immunoabsorption with specific monoclonal antibodies coupled to Sepharose.

Results

Induction of monocyte PCA by alloantigen-stimulated T cell clones. T cell clones derived from day 2 MLC cultures between irradiated Daudi B lymphoblasts and PBMCs were screened for the ability to induce monocyte PCA in response to the original stimulator cells. Fig. 1 illustrates representative data for the analysis of PBMCs and selected clones from the same individual. All clones used in this study had a surface phenotype of $OKT3^+$, $OKT4^+$, $OKT8^-$, and $OKM1^-$, typical of T helper/inducer cells, by indirect immunofluorescence. Two pathways for in-

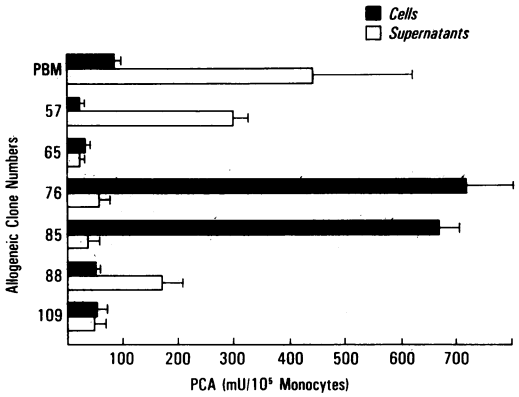

Figure 1. Induction of monocyte PCA by T clones and medium from T clones after Daudi cell stimulation. Resting T cell clones (2×10^5) or PBMCs (10^6) were cultured with irradiated Daudi cells at stimulator/responder ratios of 3:2 and 2:1, respectively, in RPMI 1640/10% FBS at 37°C for 18 h. The cells were pelleted at 400 *g*, the culture supernatants were recovered and the cells were resuspended in the original volume of medium. Stimulated cells and supernatants were then cultured separately for an additional 6 h with fresh autologous monocytes (5×10^4). Cells were recovered after centrifugation at 1,500 *g*, solubilized in 15 mM β-octylglucopyranoside, and assayed in duplicate for total cellular PCA. Results are presented as mean + standard error of the mean of duplicate reaction mixtures.

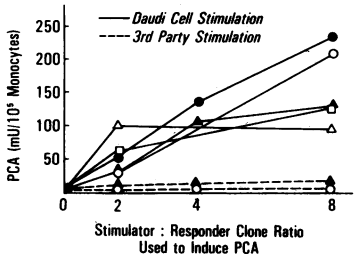

Figure 2. Effect of stimulation on magnitude of monocyte PCA response induced by T cell clones. Resting T cell clones (2×10^5) (represented by symbols ○, ●, △, ▲, and □) were cultured for 18 h at 37°C with various numbers of irradiated Daudi cells (——) or third party allogeneic cells (– – –) in RPMI 1640/10% FBS at a final concentration of 3×10^6/ml. Cell supernatants were recovered after centrifugation at 400 *g* and cultured for an additional 6 h with fresh autologous monocytes (5×10^4). The cells were then pelleted at 1,500 *g*, solubilized in 15 mM β-octylglucopyranoside, and assayed for total cellular PCA. Results are presented as the mean of duplicate reaction mixtures (SEM ≤ 0.15 × mean).

duction of monocyte PCA in response to disparate HLA-DR recognition are apparent at the level of the T cell clone. 18 h after stimulation with irradiated Daudi cells, clones 57 and 88 induced PCA exclusively through a soluble mediator. The isolated cloned cells themselves were unable to deliver the induction signal to fresh autologous monocytes. In contrast, induction of PCA in monocytes by clones 76 and 85 was demonstrated only by the stimulated cloned T cells themselves. No soluble lymphokine for PCA induction was produced by clones 76 or 85 after 18 h of stimulation with Daudi cells. PCA induction by T cell clones or their products was comparable in magnitude to that induced by supernatants from Daudi-stimulated PBMCs. Other alloantigen-responsive, proliferating T cell clones of the same surface phenotype, such as clones 65 and 109, gave no evidence of monocyte PCA-inducing activity in three rounds of analysis. These initial results utilizing T cell clones indicate that (*a*) not all alloantigen-responsive T cells that express the helper/inducer surface phenotype mediate PCA induction, and (*b*) initiation of the extrinsic coagulation protease cascade may be regulated by two separate pathways dictated by two functionally distinct subsets of T inducer clones. The inability of some alloantigen-responsive clones to deliver either of the appropriate signals for monocyte PCA expression suggests that regulation of monocyte procoagulant activity is controlled by noninclusive subsets of antigen-specific T cell clones operating through either of two collaborative pathways. The possibility that both modes of induction may be expressed by some individual clones is currently being investigated.

Requirement for appropriate antigenic stimulation of T cell clones for PCA induction. Two-stage experiments were used to determine whether T cell clones constitutively produce a PCA-inducing lymphokine and to assess the quantitative capacity of individual clones to produce the soluble inductive signal. As shown in Fig. 2, when irradiated Daudi cells and individual T cell clones were cocultured at a ratio of 2:1 for 18 h, recovered culture supernatants induced moderate levels of PCA activity in fresh autologous monocytes. At higher Daudi/T ratios the magnitude of the supernatant-induced monocyte PCA response increased in the case of each stimulated clone. No PCA-inducing lymphokine activity was found in supernatants recovered from unstimulated T cell clones or supernatants recovered from separate, autologous cultures of Daudi cells and T cell clones and then mixed. In addition, no lymphokine activity was found in supernatants recovered from T cell clones stimulated with third-party, allogeneic lymphocytes isolated by fibronectin nonadherence. PBMCs from the T cell clone donor were capable of recognizing the third-party allogeneic stimulus. Supernatants derived from 4:1 third-party lymphocytes/PBM cultures induced 525±40 mU per 10^5 monocytes in a 6-h induction assay. These data are consistent with the hypothesis that allogeneic T cells clones produce monocyte PCA-inducing lymphokine only upon restimulation with the appropriate specific alloantigen. The ability of individual T cell clones to function as lymphokine-producing inducer cells in this system appears to involve clonally restricted heterogeneity in quantitative aspects of lymphocyte triggering and lymphokine production.

Characterization of the procoagulant activity induced by allogeneic T cell clones. One-stage clotting assays were used to characterize the monocyte PCA effector molecules produced in response to alloantigen-stimulated T inducer clones. Lysates of monocytes cocultured with Daudi cells and clones 76, 85, and 88 expressed full coagulation activity in plasmas deficient in factor IX of the intrinsic coagulation pathway (Table I). The mixed cell lysates were unable to accelerate significantly the coagulation of factor VII- or X-deficient plasmas. These results were comparable to the pattern of initiation demonstrated by tissue factor using a rabbit brain thromboplastin preparation as a source of tissue factor. These data indicate that the PCA is attributable to tissue factor, the factor VII/VIIa binding cofactor that initiates this pathway, and it is induced by signals from alloantigen-stimulated T cell clones directly or indirectly by a lymphokine produced by different alloantigen-stimulated T cell clones.

Discussion

Immunologically mediated inflammatory responses are elicited by antigen-specific recognition and propagated by coupled effector responses in host defense against bacterial, viral, and pro-

Table I. Allogeneically Stimulated T Cell Clone Induction of Monocyte PCA: Requirements for Known Coagulation Proteins

Test sample	PCA clotting time*			
	Normal plasma	Factor X deficient	Factor IX deficient	Factor VII deficient
	s	*s*	*s*	*s*
Medium	183	>300	>300	280
Thrombosplastin	36	>300	38	143
Daudi + clone 76 + monocytes‡	46	>300	45	129
Daudi + clone 85 + monocytes	47	>300	49	133
Daudi + clone 88 + monocytes	67	>300	69	158

* Procoagulant activity was measured in 50 μl of test sample + 50 μl of citrated normal human platelet-poor plasma or citrated human plasmas deficient in specific coagulation proteins + 50 μl of 25 mM $CaCl_2$. Clotting times were measured from the addition of $CaCl_2$ to the formation of visible clots in borosilicate glass tubes at 37°C.
‡ Cell cultures consisted of 3×10^5 irradiated Daudi cells to 2×10^5 cloned T cells + 5×10^4 fibronectin-adherent monocytes in 0.2 ml of RPMI 1640/10% FBS. Cell pellets were recovered after 24 h of incubation, frozen, and solubilized immediately prior to testing.

tozoal infectious diseases, allografts, and many nascent tumors (29–32). Cells of the monocyte-macrophage lineage serve as principal effector cells in many inflammatory responses (33, 34). Initiation of the extrinsic protease system by monocytes and macrophages can be regulated by T lymphocytes as part of the immune response. Two types of procoagulant activity, i.e., tissue factor (35) and a unique cellular prothrombinase (36), have been described in association with activated monocyte/macrophages. Initiation of the coagulation protease cascade correlates well with delayed-type hypersensitivity responses (37) and may play a causal role in these responses. Thus, as effector cells monocyte/macrophages can generate induration characteristic of delayed-type hypersensitivity responses through the formation of fibrin (37) and various biologically active protease intermediates (38).

Antigen-specific monocyte procoagulant responses have been shown to require the collaborative participation of T lymphocytes (5, 14–17). PCA generated in human MLCs, expressed exclusively on monocytes, has been specifically identified as tissue factor (7). Expression of tissue factor has been shown to be initiated by alloantigen-specific T cells and blocked by anti-DR antibodies (7). When mixed cell populations were studied, induction of monocyte PCA in human MLCs appeared to be mediated exclusively by a soluble lymphokine produced by alloantigen-stimulated T cells, and synthesis of the lymphokine was inhibited by cyclosporin A (17). Induction of lymphokine-mediated tissue factor contrasts with the direct induction pathway of T cell–monocyte contact that has been described for PCA responses to viral antigens (6), lipopolysaccharides (2), and small, soluble immune complexes (4). An underlying difficulty in defining T cell induction pathways in mixed T cell populations results from the inability to control the sensitive regulatory interactions that operate among functional T cell subpopulations. The generation of antigen-specific T cell clones has been shown to be an incisive analytical approach to identify either T inducer or T suppressor cells (39, 40) and to provide homogeneous cellular reagents for further elucidation of the cellular events and regulatory molecules involved in T cell regulation of monocyte effector cell functions.

We report here on the identification of two collaborative pathways for inducing tissue factor expression that are displayed by alloantigen-responsive T inducer clones from one-way MLCs. We have previously observed that cell lines derived from short-term (1- or 2-d) MLCs between Daudi cells and PBMCs contained T cells which are capable of inducing monocyte PCA in response to Daudi stimulation (data not shown). In contrast, monocyte PCA inducer activity was not observed in T cell lines derived from longer-term (day 5–11) MLCs. Proliferating T cell clones were, therefore, isolated from day 2 MLC lymphoblasts in soft agar cultures with irradiated Daudi cells. Developing clones were screened for the ability to induce monocyte PCA in response to Daudi stimulator cells. T inducer and T noninducer clones were identified in initial functional screenings. T inducer clones were examined further to determine the mechanism by which they induced monocyte PCA, i.e., whether via the release of a lymphokine(s) or by direct T cell-associated induction of PCA in monocytes. Both types of T cell induction pathways were observed in selected T cell clones of typical inducer surface phenotype $OKT3^+$, $OKT4^+$, $OKT8^-$, $OKM1^-$.

These results suggest several regulatory issues which may underlie the collaborative generation of monocyte procoagulant responses. The absence of a clearly demonstrable direct T cell-associated induction pathway in mixed cell cultures may reflect the selective action of T suppressor cells or their products on this pathway. Further studies on alloreactive T cell clones may identify suppressor T cells in clone-mixing experiments. Indeed, the existence of bacterial endotoxin-driven T suppressor cells for PCA has recently been reported (41). The PCA response induced in monocytes by the cell-contact type of T inducer clone was comparable in magnitude to that induced by the medium from lymphokine-producing T inducer clones. It is conceivable that the T subset that produces the inducing lymphokine may be represented at a much higher frequency in PBMCs and may, thereby, account for the majority of T inducing activity in whole mixed cell populations in MLC using peripheral blood lymphoid cells.

Manipulation of the stimulator/responder ratio showed that T inducer clones generally produced a stronger lymphokine induction signal when stimulated by increasing numbers of the alloantigen-bearing stimulator cells. None of the clones studied produced the lymphokine signal constitutively, i.e., without Daudi cell restimulation and even in the presence of IL-2 (data not shown). In addition, production of the inductive lymphokine by T cell clones was not elicited by stimulation with allogeneic third-party lymphocytes. These results demonstrate that monocyte procoagulant activity is initiated by alloantigen-specific T inducer clones in response to restimulation with the appropriate allo-DR epitopes. Coagulation generated by either pathway of T cell clone induction was dependent on coagulation factors VII and X (but not IX), confirming that the induced monocyte PCA was the cellular tissue factor protein, i.e., the cofactor required for initiation of the extrinsic protease cascade.

Questions remain as to the role of each of these functional T inducer pathways in extravascular induction of PCA, i.e., in allografted tissue and in peripheral lymphoid tissue. Dual-inductive T cell clones may also exist as another subset of regulatory T cells, mediating their effects through both lymphokine pro-

duction and membrane signaling. The recent report of monocyte-derived interleukin 1 (IL-1) induction of tissue factor on endothelial cells (42) and speculation about the existence of a membrane form of IL-1 (43) illustrate the complexities that must be addressed at the cellular and molecular level in order to understand the cellular pathways and regulation of the procoagulant response. The generation of antigen-specific T inducer cell clones is an incisive approach to understanding effector cell regulation in the immune response and the pathogenesis of immunologic lesions. The initial findings described here demonstrate the value of T cell cloning for further investigations of tissue factor induction in human monocytes.

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