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Regulation of Endothelial VCAM-1 Expression in Murine Cardiac Grafts

Roles for TNF and IL4

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The* in vivo *mechanisms of vascular endothelial activation and VCAM-1 expression were studied in murine heterotopic cardiac grafts. Preliminary studies demonstrated that cardiac allograft endothelia develop reactivity with MECA-32 monoclonal antibody (MAb) and M/K-2 (anti-VCAM-1) MAb within 3 days of transplantation, whereas cardiac isografts develop MECA-32 reactivity but no M/K-2 reactivity. Additional studies demonstrated that a single treatment of cardiac isograft recipients with the anti-CD3 MAb 145-2C11 induces VCAM-1 expression on isograft microvascular endothelia within 24 hours. We have used this experimental system to identify the cytokines responsible for expression of VCAM-1 and MECA-32 MAb reactivity on graft vascular endothelia. We report that the expression of VCAM-1 on isograft endothelia that was induced with anti-CD3 MAb was blocked by simultaneous treatment with either pentoxifylline, soluble tumor necrosis factor (TNF) receptor (TNFR:Fc), anti-IL4 MAb, or soluble IL4R, but not by anti-IFN-γ MAb. Alternatively, a similar pattern of isograft endothelial VCAM-1 expression was stimulated in the absence of anti-CD3 MAbs with a single injection of human recombinant TNF-α, or with recombinant murine IL4 provided as IL4/anti-IL4 MAb complexes. In addition, the IL4-induced VCAM-1 expression was completely blocked by a single intravenous treatment of the isograft recipients with TNFR:Fc. This suggests that high concentrations of TNF-α can stimulate endothelial VCAM-1 expression, but these concentrations are apparently not achieved in cardiac isografts. In the absence of an inducing agent such as anti-CD3 MAb, the stimulation of VCAM-1 expression with exogenous IL4 may reflect functional interaction between endogenous TNF and exogenous IL4, as suggested by the blocking experiments with TNFR:Fc. Although cardiac isograft endothelia normally develop reactivity with MECA-32 MAb within 3 days of transplantation, treatment of cardiac isograft recipients with anti-CD3 MAb accelerated MECA-32 reactivity to within 24 hours of transplantation. This accelerated expression can be experimentally manipulated in the same way as M/K-2 reactivity, suggesting that similar mechanisms may control the development of these two inflammatory endothelial phenotypical markers, despite their differential expression in cardiac isografts and allografts. (Am J Pathol 1995, 146:989–998)

During graft rejection, like other inflammatory responses, the graft vascular endothelia develop new functional abilities to facilitate the developing inflammatory response. Among the endothelial changes is the altered expression of a variety of cell surface molecules involved in cellular adhesion, antigen presentation, or physiology function. In previous studies, we established that the microvascular endothelia of murine cardiac allografts, but not isografts, are induced

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to express the inflammatory adhesion molecule, VCAM-1, within 3 days of transplantation.[1] Similar induction of vascular VCAM-1 expression has been reported in human cardiac allografts.[2–4] Our studies also indicated that this VCAM-1 expression was associated with local T-cell activation and lymphokine production.[1] In subsequent studies, we demonstrated that treatment of cardiac isograft recipients with the anti-CD3 monoclonal antibody (MAb) 145-2C11 causes the unusual expression of VCAM-1 on isograft microvascular endothelia.[5] Presumably, this is a consequence of systemic cytokine release that accompanies the polyclonal T-cell activation caused by treatment with anti-CD3 MAb.[6]

We have also studied the expression of an endothelial epitope recognized by the MECA-32 MAb, on rejecting cardiac allografts. Unlike VCAM-1, this epitope is expressed on large-vessel microvascular endothelia in both cardiac isografts and cardiac allografts within 3 days of graft implantation.[1] Hence, expression of this epitope indicates the local development of an inflammatory response upon which alloantigen-induced inflammatory responses can be superimposed. The function of the molecule bearing the MECA-32-reactive epitope is currently unknown. Treatment of cardiac isograft recipients with anti-CD3 MAb causes the accelerated expression of MECA-32 reactivity by cardiac isograft microvasculature.

In this report, we continue our studies in these experimental systems in an effort to identify the cytokines that promote vascular VCAM-1 expression and MECA-32 MAb reactivity in cardiac grafts.

Materials and Methods

Mice

Female DBA/2 (H-2^d), C57BL/6 (H-2^b), and BALB/c (H-2^d) 5- to 6-week-old mice, all pathogen-free, were purchased from Harlan Sprague Dawley (Indianapolis, IN). Mice were maintained in the Ohio State University vivarium in accordance with National Institutes of Health guidelines.

Monoclonal Antibodies

Anti-CD3 is a hamster immunoglobulin G (IgG) MAb produced from hybridoma 145-2C11, which is reactive against the CD3 ϵ chain of the murine TCR.[7] The MAb was purified by ammonium sulfate precipitation followed by ion exchange chromatography on DE52. MECA 32 is a rat MAb (IgG2a), which was raised in rats immunized with mouse lymphoid stromal tissue. This antibody was a gift from Dr. Eugene Butcher, and recognizes an antigen that is present on murine lymphoid tissue endothelia[8] and an inducible endothelial antigen on murine cardiac grafts.[9] M/K-2 MAb is a rat IgG1 generated by immunizing rats with murine bone marrow stromal cells. This antibody recognizes a murine bone marrow stromal and endothelial cell adhesion molecule, identified as VCAM-1,[10,11] and is available from the American Type Culture Collection (Rockville, MD). Purified anti-mouse IFN-γ monoclonal antibody produced in *Escherichia coli* (clone designation XMG1.2) is a rat IgG1 obtained from PharMingen (San Diego, CA). It was diluted in PBS and the mice were injected i.v. with 500 µg. The MAb 11B11 is a rat anti-mouse IL4 MAb (IgG1). The hybridoma was obtained from American Type Culture Collection, and mice were injected i.v. with ascites (produced in BALB/c nu/nu mice) containing 500 µg MAb.

Reagents

The methylxanthine derivative pentoxifylline (3,7-dimethyl-1[5-oxohexyl]-xanthine) (PTX), was the kind gift of Hoechst-Roussel Pharmaceuticals, Inc. (Somerville, NJ). PTX was diluted in saline and mice were injected with 2 µg. i.v.[12] Purified, recombinant murine IL4 was provided by Immunex Research and Development Corp. (Seattle, WA). The IL4 was diluted in appropriate buffer containing carrier protein (BSA), and mice were injected i.v. with 10 µg or 20 µg every 6, 8, and 12 hours. Soluble murine IL4 receptor (sIL4R) was provided by Immunex Research and Development Corp. and contained 6 pg LPS/µg protein. The sIL4R was diluted in PBS, and the mice were injected subcutaneously with 100 µg. Soluble human TNF receptor linked to the Fc portion of human IgG1 (TNFR:Fc) was provided by Immunex Research and Development Corp.[13,14] TNFR:Fc was diluted in PBS with a carrier protein (BSA), and the mice were injected subcutaneously with 100 µg. All Immunex protein contained $<$10 pg/µg or protein. Recombinant human TNF-α was obtained from Genzyme Corp., Cambridge, Massachusetts. This reagent is effective for murine cells, and was diluted in PBS before i.p. injection into mice (100 to 500 µg/mouse).

Treatment of Mice with Anti-CD3 MAb

Mice were treated with a single i.v. injection of 200 µl of saline containing 10 or 20 µg of purified 145-2C11 Ig, as described elsewhere.[5,15]

Murine Cardiac Transplantation

Heterotopic cardiac transplantation was performed as described by Corry et al.[16] The native hearts from donor mice were harvested after heparinization of the animals, and placed in iced lactated Ringer's solution while the recipient mice were prepared. The donor hearts were anastomosed to recipient abdominal aorta and vena cava using microsurgical techniques. Upon re-establishment of blood flow the transplanted hearts resumed spontaneous contractions. In this stain combination, cardiac allografts undergo acute rejection with loss of function by day 10 to 12 after transplant, whereas cardiac isografts function indefinitely.

Immunoperoxidase Staining Techniques

Various murine tissues were harvested 24 to 48 hours after treatment with anti-CD3 MAb, after cervical dislocation of the recipient. Transplanted hearts were immediately embedded in Tissue Tek ornithine carbamoyltransferase (Miles, Inc., Elkhart, IN) compound, snap frozen in supercooled isopentane, and stored at −80 C. 6-μ sections were fixed for 5 minutes in acetone at 4 C and stained using standard avidin-biotin complex immunoperoxidase techniques.

All antibodies were used as undiluted tissue culture supernatants. Control sections were incubated with a 1:5 dilution of normal rat serum as the primary antibody. For endothelial staining, the secondary antibody was a biotinylated goat anti-rat IgG, which was followed by a streptavidin-horseradish peroxidase conjugate and developed in 3-amino-9-ethylcarbazole, all provided in kit form (Histo-Probe anti-rat Ig staining kit, Tago, Inc., Burlingame, CA). Sections were incubated for 30 minutes with each reagent at 37 C in a humidified chamber and developed with the chromogen 3-amino-9-ethylcarbazole (0.2% in 10% dimethyl formamide, 0.02 mol/L acetate buffer, pH 5.2, with 0.001% H_2O_2) (Sigma Chemical Co., St. Louis, MO).

Histopathology and Scoring of Slides

The immunoperoxidase-stained tissue sections were reviewed in blinded fashion by one of the authors (SDB). Slides for all of the tissues were reviewed by a minimum of two of the authors. Any discrepancies caused the procedure to be repeated. There were no significant differences between these assessments.

Four magnifications were used (×4, ×10, ×20, and ×40) for immunohistological analyses, and the immunohistochemical results were scored semiquantitatively based upon the following criteria: 0 absent, 1+ weak, 2+ present. Separate analyses were performed for large vessels and microvasculature.

Results

Interference with TNF

We previously demonstrated that treatment of cardiac isograft recipients with an anti-CD3 MAb (145-2C11) caused the expression of inflammatory adhesion molecules, including VCAM-1, on the cardiac microvascular endothelia.[5] Presumably, this is due to cytokine secretion induced by 145-2C11 treatment.[15] The studies of others have implicated TNF and IL4 as candidates for this activity.[17,18] To determine whether TNF is involved in 145-2C11-induced VCAM-1 expression, DBA/2 mice were transplanted with heterotopic cardiac isografts, injected i.v. immediately after transplant with 10 μg of 145-2C11 MAb, and i.p. with 100 μg TNFR:Fc. The cardiac isografts were harvested 24 hours later and tested immunohistochemically for endothelial VCAM-1 expression. As shown in Figure 1, cardiac isografts do not express endothelial VCAM-1 (Figure 1A) unless the graft recipient is treated with 145-2C11 MAb (Figure 1C). Under these conditions, VCAM-1 expression is very similar to the expression that normally develops in cardiac allografts (Figure 1B). In contrast, the native hearts of the treated isograft recipients develop endothelial VCAM-1 expression only in large arteries (data not shown). Treatment of isograft recipients with 145-2C11 MAb also caused an accelerated endothelial expression of MECA-32 reactivity, which is normally observed on cardiac isograft endothelial about 72 hours after transplant, but could now be detected within 24 hours (Table 1). Furthermore, treatment of the isograft recipient with 145-2C11 MAb plus TN-FR:Fc completely blocked both the MAb-induced expression of VCAM-1 (Figure 1D and Table 1) as well as the accelerated development MECA-32 reactivity (Table 1) on cardiac isograft vascular endothelia. These data indicate that TNF can play a role in the endothelial development of VCAM-1 expression and MECA-32 reactivity in cardiac grafts.

Interference with IL4

To determine whether IL4 is involved in the 145-2C11-induced VCAM-1 expression, similar experiments were performed, except that 500 μg of 11B11 (anti-IL4 MAb) i.v. or 100 μg sIL4R i.p. were used in place of the TNFR:Fc. As shown in Table 1, both anti-IL4 MAb and SIL4R blocked 145-2C11-induced VCAM-1

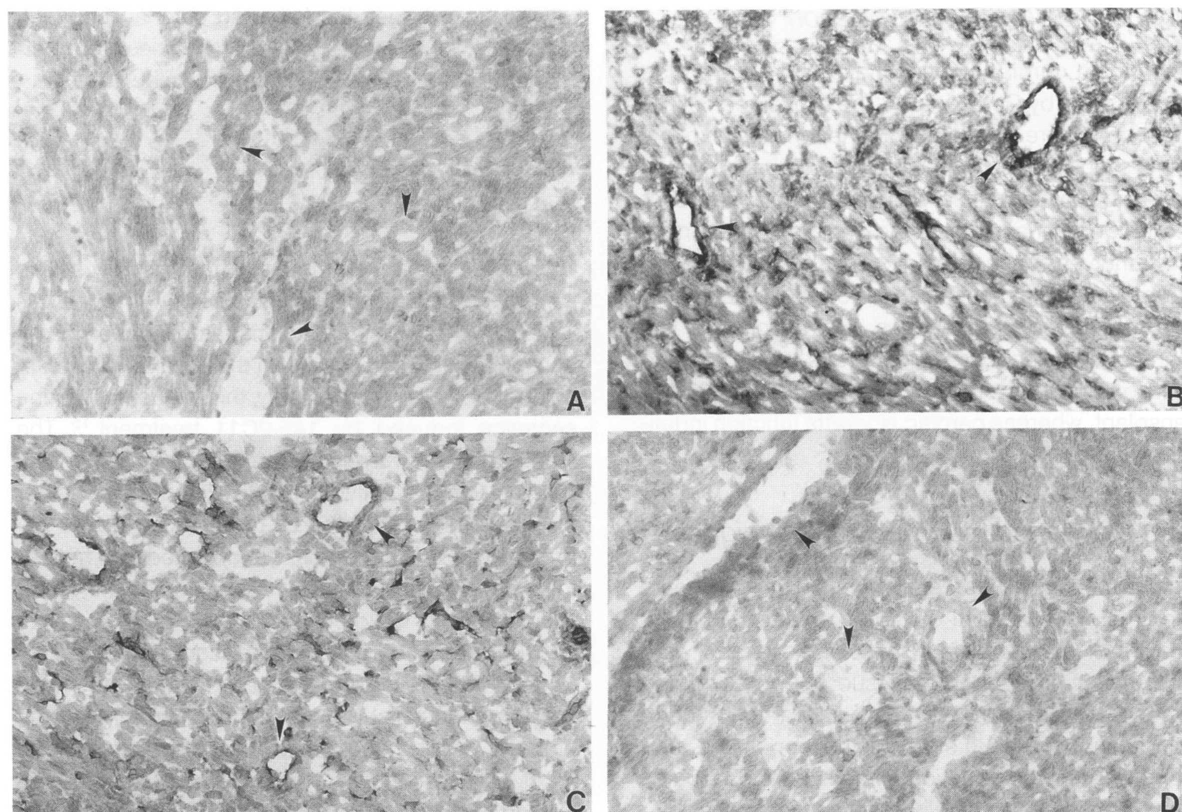

Figure 1. *Immunoperoxidase reactivity with M/K2 MAb in cardiac tissues.* (**A**) *DBA/2 → DBA/2 isograft 24 hours after transplantation (50×).* (**B**) *5-day DBA/2 → C57Bl6 cardiac allograft (50×)* (**C**) *DBA/2 → DBA/2 isograft 24 hours after transplantation and treatment of isograft recipient with 145-2C11 (50×).* (**C**) *DBA/2 → DBA/2 isograft 24 hours after transplantation and treatment of isograft recipient with 145-2C11 plus TNFr (50×). For reference, arrows identify vascular elements in these tissues.*

Table 1. *Modulation of Anti-CD3 Effects in Murine DBA/2 → DBA/2 Cardiac 24-Hour Isografts*

MAb	Treatment	VCAM-1	MECA-32	Infil	Symptoms
		Absent	Absent	Absent	Absent
CD3		Present	Present	Present	Present
CD3	2 µg PTX	Absent	Weak	Absent	Absent
CD3	100 µg TNFR:Fc	Absent	Absent	Absent	Absent
CD3	500 µg IL4 MAb	Absent	Absent	Absent	Absent
CD3	100 µg sIL4R	Absent	Absent	Absent	Absent
CD3	500 µg IFNg MAb	Present	Weak	Weak	Absent
CD4		Absent	Weak	Absent	Absent

in the cardiac isografts. They also blocked the endothelial expression of MECA-32 MAb reactivity (Table 1). Similar i.v. amounts (500 µg) of a control antibody, anti-IFN-γ MAb, did not interfere with MAb-induced expression of these endothelial surface molecules (Table 1).

Other Effects on αCD3 MAb-Induced Pathology

In addition to their influence on endothelial adhesion molecule expression, PTX, TNFR:Fc, anti-IL4 MAb, and sIL4R each eliminated the edema, cellular infiltration and pathological symptoms (quaking, piloerection, diarrhea, etc.) associated with 145-2C11 MAb treatment. Treatment with the negative control anti-IFN-γ MAb had no effect on MAb-induced endothelial adhesion molecules' expression and edema. Interestingly, the anti-IFN-γ MAb reduced both endothelial MECA-32 MAb reactivity and cellular infiltration, and eliminated pathological symptoms, as has been reported by others.[19] It should be noted that none of the endothelial effects observed with anti-CD3 MAb treatment were observed with anti-CD4

MAb treatment (Table 1), despite the fact that this MAb has profound effects on allograft responses *in vivo*.[20]

Effects of TNF-α

To determine whether TNF-α could replace 145-2C11 for induction of VCAM-1, we performed initial experiments in which DBA/2 mice received a cardiac isograft and were injected with 500 μg TNF-α i.p. These mice developed lethargy, quaking, and piloerection within 12 hours of treatment, and this condition continued to worsen until the tissues were harvested 24 hours after treatment. These animals exhibited endothelial VCAM-1 in the large vessels and microvessels of both the isograft and the native heart (Table 2), indicating strong, systemic VCAM-1 induction by TNF-α. In subsequent experiments, progressively lower doses of TNF-α were used, until at 100 μg, the collateral symptoms were no longer apparent and the VCAM-1 inductive effects were primarily confined to the isograft (Table 2, Figure 2A). Treatment of isograft recipients with exogenous TNF also caused accelerated expression of endothelial MECA-32 reactivity (data not shown). These experiments indicate that TNF-α, especially at high doses, is a potent *in vivo* inducer of endothelial VCAM-1 expression and MECA-32 reactivity.

Effects of IL4

To determine whether IL4 could replace 145-2C11 treatment for induction of VCAM-1 expression on cardiac isograft endothelia, cardiac isograft recipients were treated for 24 hours with i.v. injections of recombinant murine IL4. Several treatment protocols were used, including 10 μg IL4 every 12 hours, 20 μg IL4 every 12 hours, and 20 μg IL4 every 6 hours. As shown in Table 2, none of these protocols was able to induce detectable VCAM-1 expression in the cardiac isografts. Maliszewski and coworkers[21,22] have reported that complexes of IL4 and anti-IL4 MAb of IL4 and SIL4R (11B11) increase the magnitude and du-

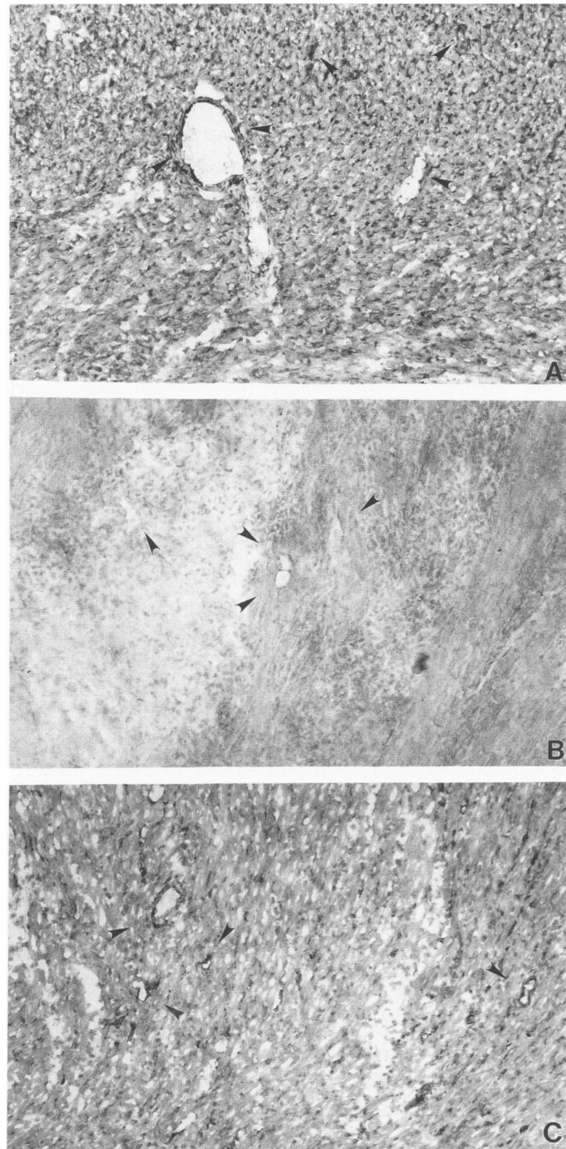

Figure 2. *Immunoperoxidase reactivity with M/K2 MAb in cardiac tissues.* (**A**) *DBA/2→ DBA/2 isografts 24 hours after transplantation and treatment of the isograft recipient with TNF-α (25×)* (**BC**) *DBA/2→ DBA/2 cardiac native heart of cardiac isograft recipient 24 hours after transplantation and treatment of the recipient with anti-IL/4 MAb/IL4 (25×).* (**C**) *DBA/2→DBA/2 isograft 24 hours after transplantation and treatment of the isograft recipient with anti-IL4 MAb/IL4 (25×). For reference, arrows identify vascular elements in these tissues.*

Table 2. *Endothelial VCAM-1 Induced by IL4 in Murine DBA/2→ DBA/2 Cardiac 24-Hour Isografts*

	IL4		IL4 MAb	Isograft		Native	
n	μg	hours	(μg)	ART	MV	ART	MV
3	10	12		Absent	Absent	Absent	Absent
2	20	12		Absent	Absent	Absent	Absent
2	20	6		Absent	Absent	Absent	Absent
3	20	6	500	Present	Present	Present	Absent
4			500	Absent	Absent	Absent	Absent

ration of IL4 effects *in vivo*. We next transplanted mice with cardiac isografts and injected them immediately with 500 µg 11B11 MAb plus 20 µg IL4. Additional injections of IL4 and anti-IL4 MAb were made at after-transplant hours 6, 12, and 18. Twenty-four hours after transplantation, the cardiac isografts and native hearts were recovered and tested for endothelial VCAM-1 expression.

As shown in Table 3 and Figure 2C, the cardiac isografts from these anti-IL4 MAb/IL4-treated mice expressed VCAM-1 on the endothelia of the microvasculature, large arteries, and large veins. In contrast, the native hearts from these mice expressed endothelial VCAM-1 only in large arteries, and occasionally in large veins, but not in the microvasculature (Table 3). Cardiac isograft recipients treated only with the 11B11 MAb did not display any endothelial VCAM-1. Thus IL4, when "protected" by anti-IL4 MAb, can induce microvascular endothelial VCAM-1 expression in 24-hour cardiac isografts, but not in the native hearts of the cardiac allograft recipients. It can also induce large-vessel endothelial expression of VCAM-1 in both the cardiac isografts and the native hearts. A similar distribution of accelerated MECA-32 MAb reactivity was also observed in these treated isograft recipients (see Table 5).

TNF/IL4 Interactions

The differential expression of VCAM-1 in cardiac isografts and native hearts of isograft recipients suggests that the isograft microvascular endothelia are hypersensitive to exogenous TNF-α and IL4. The mRNA for TNF-α can be detected in isografts within 24 hours of implantation,[23] suggesting early TNF-α production at levels that do not induce VCAM-1 expression. The hypersensitivity of isografts to exogenous TNF-α may merely reflect the combined effects of endogenous and exogenous TNF-α on endothelial behavior. IL4 mRNA is not normally detected in cardiac isografts.[23] Both *in vitro* and *in vivo* TNF and IL4 synergize for endothelial VCAM-1 expression.[17,18] The hypersensitivity of cardiac isografts to exogenous IL4 may reflect the *in vivo* synergy between the exogenous IL4 and the endogenous TNF-α.

To test this, DBA/2 mice were transplanted with cardiac isografts and treated immediately with TNFR:Fc, anti-IL4 MAb and IL4. Additional injections of IL4 and anti-IL4 MAb were made at after-transplant hours 6, 12 and 18. Twenty-four hours after transplantation, the cardiac tissues were harvested and tested for endothelial VCAM-1 expression. As shown in Table 4, a single treatment of the allograft recipient with TNFR:Fc at the time of transplantation abolished the ability of anti-IL4/IL4 to promote endothelial VCAM-1 expression in cardiac isografts. Interestingly, the TNFR:Fc did not interfere with the anti-IL4/IL4-induced expression of VCAM-1 by the arterial endothelia of the native heart in the isograft recipient, suggesting that the IL4 was sufficiently concentrated to stimulate arterial endothelia, but not transplant microvascular endothelia without the help of endogenous TNF-α.

A similar pattern of results was observed for the expression of endothelial MECA-32 reactivity (Table 5), except that exogenous IL4 induces MECA-32 reactivity on both the microvasculature and large vessels of the native heart in a TNF-α-independent manner. This suggests that expression of the endothelial epitope that reacts with MECA-32 MAb may be regulated in a somewhat similar fashion to VCAM-1.

Discussion

Previous studies by Ferran et al[15] have demonstrated that i.v. treatment of mice with a murine CD3-reactive MAb, 145-2C11, caused systemic, polyclonal T-cell activation and the release of multiple cytokines, including IFN-γ, TNF, IL1, IL2, IL3, IL6, and GM-CSF. A similar response occurs in humans after treatment with OKT3.[24] Many studies suggest that the expression of endothelial adhesion molecules, including VCAM-1, is regulated by cytokines. For example, *in vitro* studies demonstrated that IL1, TNF-α and IL4 can each induce VCAM-1 expression on cultured human umbilical vein endothelia cells.[25] Similar effects of these cytokines have been observed *in vivo* by

Table 3. *Endothelial VCAM-1 Induced by IL4 in Murine DBA/2 → DBA/2 Cardiac 24-Hour Isografts*

n	IL4 (µg)	IL4 MAb (µg)	sTNFR (µg)	Isograft		Native	
				ART	MV	ART	MV
3				Absent	Absent	Absent	Absent
2	20			Absent	Absent	Absent	Absent
3	20	500		Present	Present	Present	Absent
4			100	Absent	Absent	Absent	Absent
3	20	500	100	Absent	Absent	Present	Absent

Table 4. *Endothelial VCAM-1 Induced by TNF-α in Murine DBA/2 → DBA/2 Cardiac 24-Hour Isografts*

n	TNF-α	Isograft		Native	
		ART	MV	ART	MV
3	None	Absent	Absent	Absent	Absent
2	500 μg	Present	Present	Present	Present
2	300 μg	Present	Present	Present	Present
2	200 μg	Present	Present	Present	Present
3	100 μg	Present	Present	Weak	Absent

Briscoe and colleagues,[17] who found that TNF induced microvascular VCAM-1 expression, and that IL4, which alone was ineffective, synergized with TNF to promote VCAM-1 expression when injected into baboon skin. These observations suggest TNF and IL4 as cytokines that may be involved in endothelial responses at graft sites. However, it is important to remember that the endothelial cells in different vascular beds may respond differently to various cytokines. For example, Swerlick et al[26] demonstrate that cultured human dermal microvascular endothelial cells express VCAM-1 in response to TNF-α, but not IL1 or IL4; whereas cultured umbilical vein endothelial cells express VCAM-1 in response to all three cytokines. This indicates that the mechanisms that regulate endothelial behavior are not universal, and may differ according to endothelial location. Hence, inflammatory endothelial responses that occur in different tissues must be independently defined. To date, no studies have defined the cytokines that are operative *in situ* on graft vascular endothelia.

To approach this question, we reasoned that the systemic bolus of cytokines caused by injection of anti-CD3 should cause systemic endothelial activation, reflected by changes in vascular endothelial surface phenotype. In a previous report,[1] we demonstrated that the vascular endothelia of murine heterotopic cardiac grafts can display several stable phenotypes. For example, cardiac allograft endothelia begin to express the adhesion molecule, VCAM-1, within 72 hours of transplantation. Cardiac isograft endothelia do not display VCAM-1, but undergo transient inflammation that is characterized by the expression of another endothelial epitope, identified with MECA-32 MAb, within 72 hours of transplantation. When we tested the hypothesis, we found that treatment of mice with 145-2C11 MAb caused VCAM-1 expression and MECA-32 MAb reactivity on endothelial cells of large arterial vessels of all organs investigated.[5] Further, we found that transplanted cardiac isografts were hypersensitive to the anti-CD3 treatment in that the isografts, but not the native hearts of transplant recipients, also developed microvascular endothelial expression of VCAM-1 and MECA-32 reactivity. The reasons for this hypersensitivity were not clear, but the nature and intensity of the responses provided an experimental system for the identification of cytokines that operate *in vivo* to cause the expression of VCAM-1 and other molecules on the graft vascular structures.

In this report, we provide evidence that TNF is involved in the induction of cardiac graft endothelial VCAM-1 expression. Stimulation of cardiac isograft recipients with anti-CD3 MAb causes VCAM-1 expression on the isograft microvascular endothelia that is blocked by simultaneous treatment with TNFR:Fc (Table 1, Figure 1D). Similarly, treatment of cardiac allograft recipients with TNFR:Fc eliminates the VCAM-1 expression that normally develops on allograft microvascular endothelia (S. Bergese, Submitted for publication). A single i.v. injection of cardiac isograft recipients with high doses (>200 μg/mouse) of recombinant TNF-α causes VCAM-1 expression on both graft and nongraft endothelia within 24 hours (Table 2, Figure 2A). At lower doses (100 μg/mouse), TNF-induced VCAM-1 expression is generally limited to graft vascular structures (Table 2), illustrating a hypersensitivity of isograft tissues to this effect of TNF-α. TNF mRNA can be detected by RT-PCR in both DBA/2 → DBA/2 cardiac isografts and DBA/2 → C57BL/6 cardiac allografts within 24 hours of transplantation,[23] yet VCAM-1 expression is observed only in cardiac allografts.

Based on these observations, we suggest that TNF-α is produced in both isografts and allografts in concentrations that are too low to promote local immunoperoxidase-detectable VCAM-1 expression. When limiting amounts of exogenous TNF-α are provided, the combined concentration of exogenous and endogenous TNF-α is sufficient to promote VCAM-1 expression in grafts, but not native hearts (which lack endogenous TNF-α). Higher concentrations of exogenous TNF-α overcome this endogenous deficit and promote endothelial VCAM-1 expression in nongrafted tissues, including the native heart. Another cytokine, IL4, also appears to be involved in graft endothelial VCAM-1 expression. The induction of graft endothelial VCAM-1 by treatment of the recipient with anti-CD3 MAb can be blocked by simultaneous injection with anti-IL4 MAb or soluble IL4R (Table 1). Injection of cardiac isograft recipients with recombinant IL4 promotes VCAM-1 expression on the microvascular endothelia of the graft, but not the native heart (Table 3, Figure 2C). Interestingly, simultaneous treatment of isograft recipients with IL4 and TNFR:Fc blocks isograft endothelial VCAM-1 expression (Table 4), suggesting a role for endogenous TNF-α in this

Table 5. *Endothelial MECA-32 Induced by IL4 in Murine Cardiac Isografts*

n	IL4 (μg)	IL4 MAb (μg)	sTNFR (μg)	Isograft		Native	
				ART	MV	ART	MV
3				Absent	Absent	Absent	Absent
2	20			Absent	Absent	Absent	Absent
3	20	500		Present	Present	Present	Present
4			100	Absent	Absent	Absent	Absent
3	20	500	100	Absent	Absent	Present	Present

response. Using RT-PCR, it is possible to detect IL4 mRNA in cardiac allografts 3 to 4 days after transplantation, whereas IL4 mRNA is not detectable at any time in cardiac isografts.[23,27] Hence, VCAM-1 expression correlates with IL4 mRNA production, but not TNF-α mRNA production in cardiac grafts.

Based on these data, we suggest that cardiac isografts fail to express endothelial VCAM-1 because the level of endogenous TNF-α production is insufficient, and IL4 production is absent. When exogenous IL4 is provided, it synergizes with endogenous TNF-α to promote VCAM-1 expression on isograft microvascular endothelia, but not on the microvascular endothelia of native hearts (which lack endogenous TNF-α). This explains why TNFR:FC blocks IL4-induced VCAM-1 expression on isografts vascular endothelia. Similarly, when endogenous IL4 is available, as it is in allografts after 3 to 4 days of implantation, local endothelial VCAM-1 expression develops.

Initially, we were unable to demonstrate the ability of exogenous IL4, at any of several doses and treatment schedules, to induce VCAM-1 expression on graft or recipient vascular endothelia. Presumably, this was due to the rapid metabolism of the IL4. Finkelman and colleagues have reported that the *in vivo* biological activity of IL4 can be protected by complexing IL4 with anti-IL4 MAb before injection. We used a slight variation of this approach, and treated mice with i.v. injections of 500 μg of 11B11 and 20 μg recombinant IL4, followed every 6 hours by another injection of 20 μg IL4. Only under these conditions could we observe the IL4-mediated induction of endothelial VCAM-1 expression on vascular endothelia. This confirms the observation of Finkelman[21] regarding potentiating effects of anti-IL4 MAb on IL4 activity *in vivo*, and adds the induction of endothelial VCAM to the list of these effects.

We were also interested in the regulation of the inflammatory endothelial surface molecule, identified by the MAb MECA-32. In previous studies, we observed that this molecule is expressed in both cardiac isografts and allografts within 72 hours of implantation. In the current studies, the influence of exogenous cytokines was manifest as the accelerated development MECA-32 reactivity to within 24 h of transplantation. During these studies, we found that endothelial MECA-32 MAb reactivity in cardiac grafts was influenced by TNF and IL4 in a manner similar to the induction of endothelial VCAM-1 expression. Exogenous IL4 and exogenous TNF-α both promoted endothelial MECA-32 reactivity (Table 5), and exogenous IL4 appeared to cooperate with endogenous TNF-α for this effect (Table 5). Based on these data, we suggest that endothelial MECA-32 reactivity develops in isografts either 1) when sufficient levels of TNF-α become available (about 3 days after graft implantation; these levels would be insufficient to promote VCAM-1 expression); or 2) when a second, unidentified co-stimulus becomes available around the 3rd day of implantation to synergize with endogenous TNF-α. Regulation of endothelial MECA-32 reactivity in allografts may be slightly different, as our experiments suggest that one of the TNF co-stimulatory molecules for MECA-32 reactivity in allografts may be IL4.

In our early experiments, we observed that PTX can influence inflammatory endothelial behavior in cardiac isografts (Table 1). PTX is a methylxanthine derivative with strong immunosuppressive activities. For example, PTX blocks T-cell proliferation stimulated *in vitro* by lectins, alloantigens, or mitogenic antibodies.[28] Further, the pathological effects associated with anti-CD3 MAb treatment, which are dependent on cytokines released by MAb-activated T cells,[24,29] are blocked by PTX in experimental animals.[12] The exact mechanisms by which PTX exerts its immunosuppressive effects are unknown, but *in vitro* studies suggest that PTX can interfere with mRNA and protein TNF-α production.[30] In this regard, it is not surprising that PTX blocks the expression of endothelial VCAM-1 caused by injection of anti-CD3 MAb (Table 1), given the apparent dependence of VCAM-1 expression on TNF-α.

In general, we have provided evidence that IL4 and TNF-α play important roles in the process of endothelial activation in murine heterotopic cardiac grafts. Our observations in cardiac isografts suggest that

similar mechanisms of endothelial regulation may be operative in cardiac allografts. In a subsequent communication (Bergese, Submitted for publication), we will directly address the role of IL4 and TNF in allograft endothelial responses, and will demonstrate the significance of these responses to the process of cardiac allograft rejection.

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