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Analysis of CD154 and CD40 expression in native coronary atherosclerosis and transplant associated coronary artery disease

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Abstract T cells have roles in the pathogenesis of native coronary atherosclerosis (CA) and transplant-associated coronary artery disease (TCAD). The mechanisms by which T cells interact with other cells in these lesions are not fully known. CD154 is an activation-induced $CD4^+$ T cell surface molecule that interacts with $CD40^+$ target cells, including macrophages and endothelial cells, and induces the production of pro-inflammatory molecules, including CD54 (ICAM-1) and CD106 (VCAM-1). To investigate whether CD154-CD40 interactions might be involved in the pathogenesis of CA or TCAD we performed immunohistochemical studies of CD154 and CD40 expression on frozen sections of coronary arteries obtained from cardiac allograft recipients with CA (n=10) or TCAD (n=9). Utilizing four different anti-CD154 mAb we found that CD154 expression was restricted to infiltrating lymphocytes in CA and TCAD. CD40 expression was markedly up-regulated on intimal endothelial cells, foam cells, macrophages and smooth muscle cells in both diseases. Dual immunolabeling demonstrated many $CD40^+$ cells co-expressed CD54 and CD106. The extent of CD40, CD54 and CD106 expression showed statistical significant correlation with the severity of disease and the amount of intimal lymphocytes. Together these studies demonstrate the presence of activated $CD154^+$ and $CD40^+$ cells in both CA and TCAD lesions and suggest that CD154-mediated interactions with $CD40^+$ macrophages, foam cells, smooth muscle cells and/or endothelial cells may contribute to the pathogenesis of these diseases.

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Introduction

Several lines of evidence indicate that cell-mediated immune mechanisms contribute to the inflammatory lesions characteristic of native coronary atherosclerosis (CA) and transplant-associated coronary artery disease (TCAD) [3, 7, 16, 17, 22, 24, 26, 31, 37]. For example, infiltrating intimal T cells expressing activation markers such as CD25 and MHC class II molecules are present early in the development of the vascular lesions of both diseases. Activated macrophages are commonly found in lesions of both diseases, as are cytokines associated with T-cell-dependent immune responses, including IFN-γ, IL-1 and TNF-α. Further evidence for a role of T cells in the pathogenesis of CA is the presence of $CD4^+$ T cell clones isolated from human fibroatheromatous plaques that proliferate and secrete IFN-γ when exposed to oxidized LDL [27]. Such LDL activated T cells may further the progression of native CA by activating intimal cells, including macrophages, smooth muscle cells and endothelial cells. With regard to TCAD, vascular lesions were significantly ameliorated when allografts were placed in T cell-deficient mice or animals treated with anti-CD4 or anti-IFN-γ mAbs [23, 25], suggesting that a T-cell-mediated chronic inflammatory response is responsible for progression of TCAD. Several T-cell-derived stimulatory molecules are known to interact with effector cells and serve to perpetuate inflammation.

CD154 is a 30- to 33-kDa (mol. wt.) surface molecule expressed predominantly on activated $CD4^+$ T cells, and it delivers contact-dependent signals to target cells via interactions with CD40 [1, 10, 18]. The importance of CD154–CD40 interactions in the development of humoral and cellular immune responses is well documented [2, 28]. Recently, CD40 has been detected on many different cell types, including macrophages, vascular smooth muscle cells and endothelial cells, all of which participate in

the pathogenesis of CA and TCAD [5, 6, 12, 34]. Moreover, CD154-mediated signals induce macrophages, vascular smooth muscle cells and endothelial cells to produce pro-inflammatory and pro-coagulant molecules in vitro. Ligation of CD40 on macrophages induces the production of cytokines (TNF-α, IL-1β, IL-12), chemokines (IL-8, MIP-1α), nitric oxide (NO) via induction of NO synthase II and matrix metalloproteinases [6, 14, 28, 29]. We and others have shown that CD154–CD40 interactions up-regulate intercellular adhesion molecules CD54 (ICAM-1), CD106 (VCAM-1) and CD62E (E-selectin) on endothelial cells [5, 33]. Ligation of CD40 on monocytes and endothelial cells also has pro-coagulant effects, in part mediated by tissue factor up-regulation [15, 20].

Recent studies strongly suggest that CD154–CD40 interactions participate in the pathogenesis of CA and TCAD. In this regard, administration of an anti-CD154 mAb markedly blocks the development of atherosclerosis in LDL-R KO mice fed an atherogenic diet [13]. Moreover, CD154–CD40 interactions contribute to the vasculopathy associated with allogeneic transplant rejection in mice [9]. Mach and colleagues studied CD154 and CD40 expression in atherosclerotic lesions of human carotid arteries and found marked up-regulation of CD40 expression on many cell types [12]. This group also presented evidence that in addition to T cells, endothelial cells, macrophages and smooth muscle cells express CD154 in atherosclerotic lesions, and that resting and cytokine-activated saphenous vein endothelial cells, macrophages and smooth muscle cells express CD154 in vitro [12]. Reul et al. also showed by FACS analysis that cytokine activated human umbilical vein endothelial cells express CD154 [21].

There have been no studies examining CD154 or CD40 expression in TCAD. In this study we determined the cellular distribution of CD154 and CD40 expression in TCAD. We also re-examined the cellular distribution of these molecules in native atherosclerotic lesions, specifically in CA. We found CD40 expression up-regulated on multiple cell types in TCAD and CA lesions. CD154 expression, however, was restricted to T cells in TCAD, and in marked contrast to the findings reported by Mach et al., in native atherosclerotic lesions as well. The localization of CD40 and CD154 in TCAD and CA suggests that interaction of these molecules may contribute to a T-cell-mediated inflammatory process common to both vascular diseases.

Methods

Human coronary arteries

Segments from the main left coronary artery or the proximal portion of the left anterior descending artery were obtained from the explanted hearts of 23 cardiac allograft recipients. Nine patients underwent retransplantation because they had developed severe TCAD. In these patients survival of the first allograft had ranged between 38 and 103 months. Ten patients received cardiac allografts because they had developed severe coronary artery disease and ischemic cardiomyopathy. Control coronary arteries without atherosclerotic changes were obtained from explanted hearts of 4 patients; 3 had idiopathic cardiomyopathy, and 1 a cardiac sarcoma. Portions of each vessel were snap-frozen in isopentane at –80°C, and serial sections were cut on a cryostat (Reichert Histostat) at 4 µm thickness. Sections were mounted on sialin-coated slides, air-dried, fixed in cold acetone for 1 min, in a 1:1 mixture of cold acetone/chloroform for an additional 7 min and stored at –80°C. One section from each coronary artery was fixed in 10% formalin and stained with hematoxylin and eosin for histological evaluation.

Primary antibodies

Anti-CD40 hybridoma G28.5 (IgG1) was purchased from American Type Culture Collection (Rockville, Md.). Anti-CD154 mAb clone 5C8 (IgG2a) was generated in our laboratory as previously described [10]. Both G28.5 and 5C8 mAbs were purified from ascites utilizing a protein G column (Pharmacia, Piscataway, N.J.). Additional unconjugated anti-CD154 mAbs (IgG1) were purchased from Calbiochem (San Diego, Calif.) (clone: 24–31) and Pharmingen (San Diego, Calif.) (clone: TRAP). Purified anti-CD154 mAb clone M90 (IgG1) was provided by Christopher Benjamin (Biogen, Cambridge, Mass.). PE-conjugated anti-CD154 mAb (TRAP) was obtained from Pharmingen. An IgM anti-CD40 mAb was obtained from Caltag (Burlingame, Calif.) and was used for dual immunostaining studies. Anti-CD31 and anti-CD106 mAb secreting hybridomas were purchased from the Developmental Studies Hybridoma Bank (University of Iowa). FITC-conjugated anti-CD14 mAb was obtained from Biosource (Camarillo, Calif.). Monoclonal antibodies to CD3, CD4, CD8, CD68 (Novocastra, Burlingham, Calif., all IgG1) and smooth muscle actin (SMA) (DAKO, Carpinteria, Calif., IgG2a), were used to distinguish among the various cell types of intimal plaques, including T cells (CD3, CD4 or CD8), endothelial cells (CD31), macrophages (CD68) and smooth muscle cells (SMA). Anti-CD54 (ICAM-1, IgG1) and anti-CD106 (VCAM-1, IgG1) mAbs were purchased from Chemicon (Temecula, Calif.). Isotype control mAb (Mopec 21, 22) were obtained from Sigma (St. Louis, Mo.).

Immunohistochemical studies

Frozen sections were washed in phosphate-buffered saline (PBS) and endogenous peroxidase was quenched in 0.5% hydrogen peroxide. Sections were “blocked” with 10% goat serum and aggregated human Ig (80 µg/ml) in PBS and then were incubated for 1 h with the primary indicated mAb or the respective control mAb. Frozen sections of tonsils with follicular hyperplasia were used as positive controls to determine the optimal dilution of each mAb. Primary mAb bound to target antigen was linked to biotin-labeled isotype-specific goat anti-mouse IgG1, IgG2a or IgM (Fisher Scientific, Pittsburgh, Pa.), which was then conjugated to avidin-biotin peroxidase complexes (Vector Elite Kit, Vector, Burlingham, Calif.). Peroxidase activity was detected by the chromogen (brown) 3,3’-diaminobenzidine (DAB, Vector, Burlingham, Calif.) and the sections were counterstained with Mayer’s hematoxylin (Sigma, St. Louis, Mo.).

Double-labeling immunohistochemistry was used to identify the cell types expressing CD40 and to determine the distribution of CD40 in relation to CD54 or CD106 in atherosclerotic lesions. All sections were first immunolabeled with the IgM anti-CD40 mAb. The secondary Ab was a biotinylated goat anti-mouse IgM, which was then conjugated to the avidin-biotin peroxidase complex. The chromogen used to detect the presence of anti-CD40 IgM mAb was 3,3’-diaminobenzidine (brown). The sections were then rinsed thoroughly and incubated with a second primary mAb targeting either a cell-specific marker for smooth muscle cells (SMA) or macrophages (CD68) or leukocyte adhesion molecules (CD54, CD106). All of these second primary mAbs were either IgG1 or IgG2a isotypes. The appropriate isotype-specific biotin-

ylated secondary antibody was applied and conjugated to an avidin–biotin–alkaline phosphatase complex (Vector, Burlingham, Calif.). Alkaline phosphatase activity was demonstrated by the chromogen Vector Red (Vector). Interference between the sequentially applied staining procedures was avoided by using different immunoenzymatic techniques (peroxidase vs alkaline phosphatase) and isotype-specific secondary Abs for each target antigen. Furthermore, double-labeled control sections were prepared in which one of the two primary mAbs was substituted with an isotype-matched control mAb.

Semi-quantitative analysis of lesions

The extent of the atherosclerotic lesions in each section was quantified by the degree of narrowing of the vascular lumen on a scale from 0 to 4, in which 0 indicated no narrowing, 1 less than 25%, 2 less than 50%, 3 less than 90%, and 4 over 90% luminal narrowing. Each coronary artery lesion was also scored for its content of intimal macrophages, smooth muscle cells, foam cells, endothelial cells (neovascularization) [8] and T cells, with 0 indicating absence of the respective cell type, 1 rare isolated cells, 2 small collections of cells, 3 focal dense aggregates present, and 4 dense aggregates present throughout the entire intimal lesion. Similarly, the presence of CD40, CD54, and CD106 was scored on a scale from 0 to 4, in which 0 indicates absence of the respective molecule, 1 its presence on rare cells, 2 its presence on less than 50%, 3 on less than 90%, and 4 on more than 90% of all cells [19]. The semi-quantitative analyses were performed by M.S.

Statistical analysis

Differences in histological scores among groups of specimens were analyzed using the non-parametric Kruskal-Wallis procedure. The association between variables was assessed using Spearman's correlation.

Cell culture

Primary human umbilical vein endothelial cells (HUVEC) were obtained by collagenase treatment of umbilical veins [34]. Saphenous vein endothelial cells (SVEC) were kindly provided by two sources: Dr. John Artrip (Columbia University) and Drs. Peter Libby and Francois Mach (Harvard University). All endothelial cells were cultured on plasticware that had been precoated with either gelatin or fibronectin. HUVEC and SVEC provided by Dr. Artrip were cultured in M199 media (Gibco, Grand Island, N.Y.) supplemented with 25% FCS (Summit Biotechnology, St. Collins, Conn.), 5% human serum (Gemini, Calabasas, Calif.), heparin 90 µg/ml (Sigma), endothelial cell growth factor 15 µg/ml (Collaborative Research, Bedford, MA) and 1% penicillin-streptomycin (Sigma). SVEC provided by Drs. Libby and Mach were cultured in media supplied by these investigators [13]. Monocytes were either prepared as previously described [35] or kindly provided by John Loike (Columbia University) and cultured in Isocoves Modified Dulbecco's Medium (Gibco) supplemented with 10% FCS and 1% penicillin-streptomycin. Human aortic smooth muscle cells (HAoSMC) and human coronary artery smooth muscle cells (HCaSMC) were purchased from Clonetics and cultured in Smooth Muscle Growth Media (Clonetics). To determine CD154 expression on resting or activated endothelial cells, HUVEC, SVEC, HAoSMC or HCaSMC, cells were cultured in 6-well plates until nearly confluent. Fresh media was added and the cells were cultured in the presence or absence of TNF-α (10 ng/ml) (R&D, Minneapolis, Minn.), IL-1β (5 ng/ml, R&D) or IFN-γ (1000 U/cc, Biogen) for 6 h or 24 h. The cells were collected by adding cold EDTA and scraping the plates. One million monocytes were cultured on 6-well plates in the presence or absence of cytokines and also collected by EDTA treatment and scraping. In some experiments, monocytes were cultured in Teflon-coated containers (Pierce, Rockford, Ill.). A control T cell Jurkat clone D1.1 [10] that constitutively expresses CD154 was similarly treated with EDTA prior to FACS analysis.

Cytofluorographic analysis

Single-color FACS analyses were performed as previously described [10]. Briefly, harvested cells were stained with saturating concentrations of mAbs at 4°C for 30–60 min. After washing, the cells were then stained with PE-conjugated F(ab)2' goat anti-mouse IgG for 30–60 min at 4°C prior to further washing and FACS analysis. Monocytes were analyzed by two-color FACS. The cells were first pretreated with aggregated human IgG (International Enzymes, Fallbrook, Calif.) as previously described [10] and then reacted with FITC-conjugated anti-CD14 mAb and PE-conjugated anti-CD154 mAb (TRAP) or PE-conjugated isotype control mAb. Electronic gates were placed around $CD14^+$ cells and CD154 fluorescence determined. Fluorescence intensity was measured on an FACScan cytofluorograph with Cell Quest software (Becton-Dickinson, Mountainview, Calif.).

Results

Analysis of CD154 and CD40 expression in normal coronary arteries and morphological characterization of CA and TCAD lesions

Artery segments from 4 control patients exhibited no intimal thickening or inflammation on H&E staining. Specifically, macrophages, smooth muscle cells, foam cells or lymphocytes were not present in the intima and no immunostaining was noted with any of the anti-CD154 mAbs used in this study (data not shown). CD40 immunoreactivity was present and confined to endothelial cells lining the vascular lumen of the control arteries (data not shown).

All coronary artery lesions (CA and TCAD) analyzed were complex fibroatheromatous plaques. Intimal lesions of CA consistently had a lipid core, which was frequently calcified and covered by a fibrous cap. Lymphocytes were predominantly found at the shoulder of the plaques and were frequently associated with neovascularization. Foam cells and macrophages tended to co-localize with lymphocytes. Intimal lesions of TCAD showed many more lymphocytes than those of CA. Lesions of TCAD showed concentric intimal fibroplasia and absence of lipid cores. Foam cells were present in all cases of TCAD and in 4 of the 9 cases formed large clusters with close proximity to the endothelium.

Immunohistochemical analysis of CD154 expression in CA and TCAD

In marked contrast to normal coronary arteries, which are devoid of infiltrating lymphocytes or CD154-expressing cells, both CA and TCAD lesions contained $CD154^+$ lymphocytes. $CD154^+$ lymphocytes were found in intimal lesions of both CA (Fig. 1a) and TCAD (Fig. 1b). In TCAD, adventitial lymphocytes were also strongly $CD154^+$. Lymphocytes were more numerous in TCAD

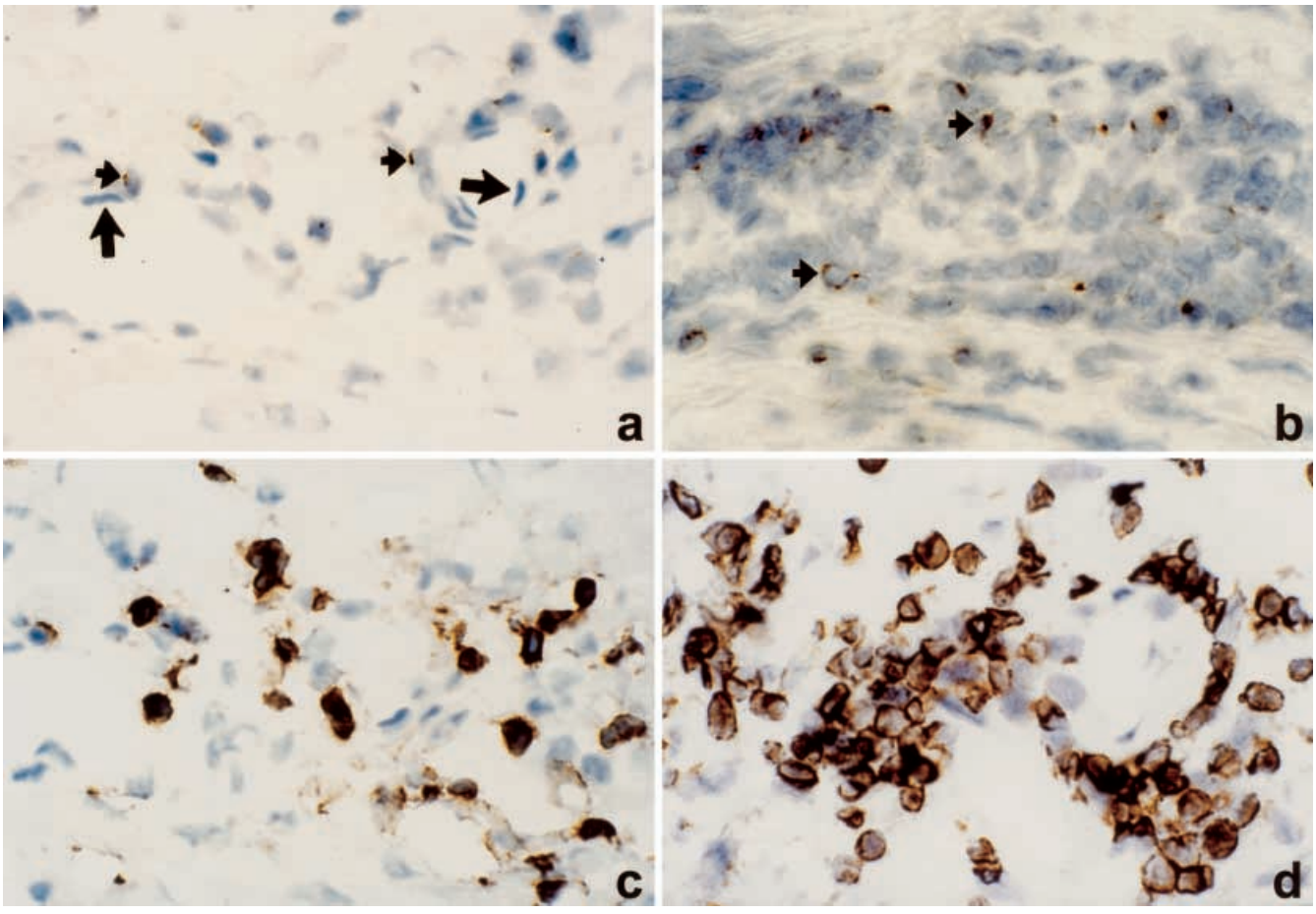

Fig. 1a-d CD154 expression in intimal lesions of native coronary atherosclerosis (CA) and transplant-associated coronary artery disease (TCAD). Serial sections of coronary arteries from patients with **a**, **c** CA or **b**, **d** TCAD, demonstrating **a**, **b** CD154 expression and **c**, **d** CD3 expression. Note that CD154 expression, as demonstrated by staining with anti-CD154 mAb 5C8 (*short arrows*), appears to be restricted to $CD3^+$ T cells while adjacent endothelial cells with spindle-shaped nuclei (*long arrow*) are CD154 negative. Similar results were obtained with all four anti-CD154 mAbs utilized in this study. There was no immunoreactivity with isotype control mAbs (not shown). Original magnification ×600

lesions (Table 1), and the number of $CD154^+$ cells was also greater in TCAD than in CA. Analysis of serial sections stained with anti-CD3 (Fig. 1c, d), anti-CD4 or anti-CD8 mAbs strongly suggested that the $CD154^+$ lymphocytes were primarily $CD4^+$ T cells. Similar findings were seen using four different anti-CD154 monoclonal antibodies (clones 5C8, TRAP, M90 and 24–31). No staining was noted with isotype control mAbs.

CD154 was not detected on luminal or neovascular endothelial cells in either CA or TCAD lesions (Fig. 2a, b). As a control, CD154 was detected on T cells in tonsil using identical staining techniques (Fig. 2c). In areas of neovascularization $CD154^+$ lymphocytes were found in close proximity to endothelial cells (Fig. 1a), which were identified by CD31 staining and morphologic features. As in endothelial cells, CD154 was not detected on smooth muscle cells or macrophages in CA or TCAD lesions.

Table 1 Semiquantitative evaluation (scale 0–4) of cell composition in intimal lesion of native coronary atherosclerosis (*CA*) and transplant coronary artery disease (*TCAD*) and the immunoreactivity for CD40, CD54 (ICAM-1), and CD106 (VCAM-1). Values are expressed as mean ±standard deviation

Intimal plaque	Control (n=4)	CA (n=10)	TCAD (n=9)
Thickness	0.3±0.5	2.1±0.9*	3.1±0.8*
CD4+ lymphocytes	0	1.3±0.9*	3.2±0.8*
CD8+lymphocytes	0	0.3±0.5	2.6±1.1*
Macrophages (CD68)	0.5±0.6	2.1±0.8*	3.8±0.4*
Foam cells	0	1.2±0.8*	2.4±1.3*
Smooth muscle cells	0.8±1	1.7±0.7	2.9±0.8*
Neovascularization	0	1.8±0.7*	2.6±0.9*
CD40	0.5±0.6	2.2±0.7*	3.3±0.9*
CD54	0.5±0.6	2.3±1.7*	3.6±0.7*
CD106	0.3±0.5	1.7±0.7*	2.9±0.9*

*P<0.05 for CA or TCAD vs controls (Kruskal-Wallis test)

Determination of CD154 expression on endothelial cells in vitro

In contrast to a previous report [12], our in situ investigation of human atherosclerotic lesions demonstrated that CD154 expression was restricted to T cells. Prior studies have suggested that resting and/or cytokine-activated endothelial cells, macrophages and smooth muscle cells also express CD154 in vitro [12, 21]. In the next series of experiments we also asked whether resting or cy-

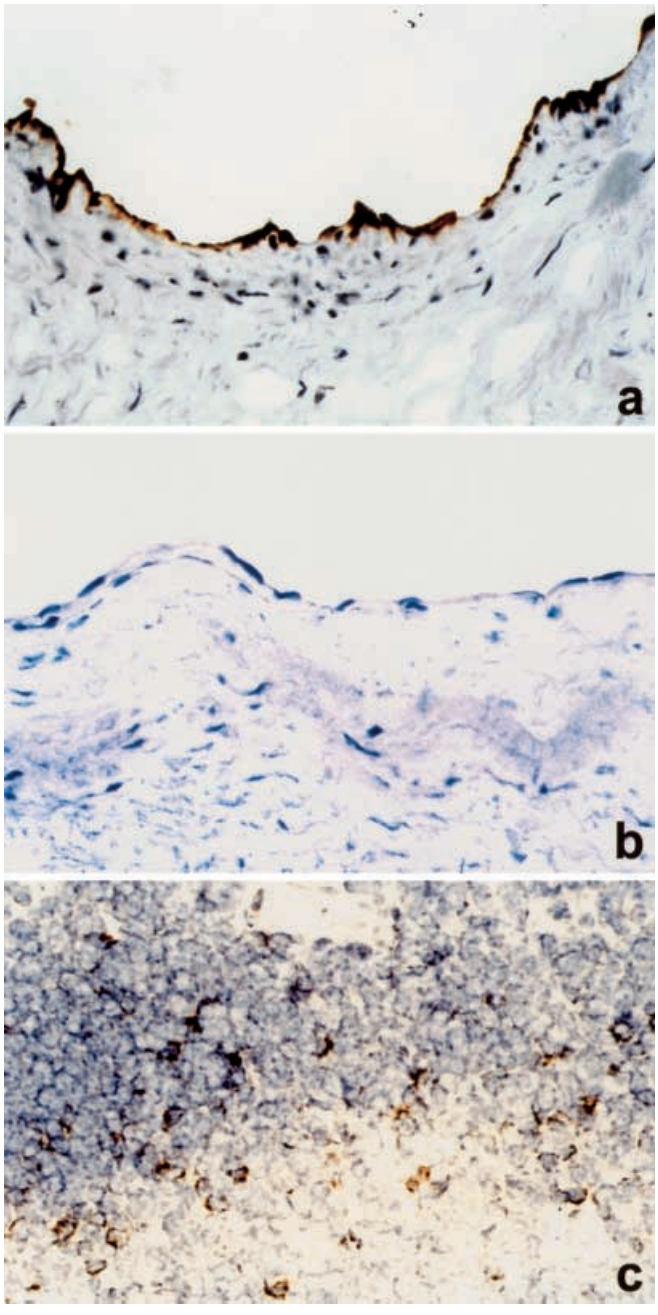

Fig. 2a-c CD154 expression in CA and TCAD is restricted to T cells. Serial sections of a CA lesion stained with **a** anti-CD31 mAb or **b** anti-CD154 mAb TRAP. Note the absence of CD154 expression on $CD31^+$ intimal endothelial cells. **c** Tonsil was used as a positive control for CD154 expression. Lesional smooth muscle cells, macrophages and foam cells were also CD154 negative. Similar results were obtained with all four anti-CD154 mAbs utilized in this study. Endothelial cells, smooth muscle cells, macrophages and foam cells in TCAD lesions also lacked CD154 expression (not shown). There was no immunoreactivity with isotype control mAbs (not shown). (Original magnification ×400)

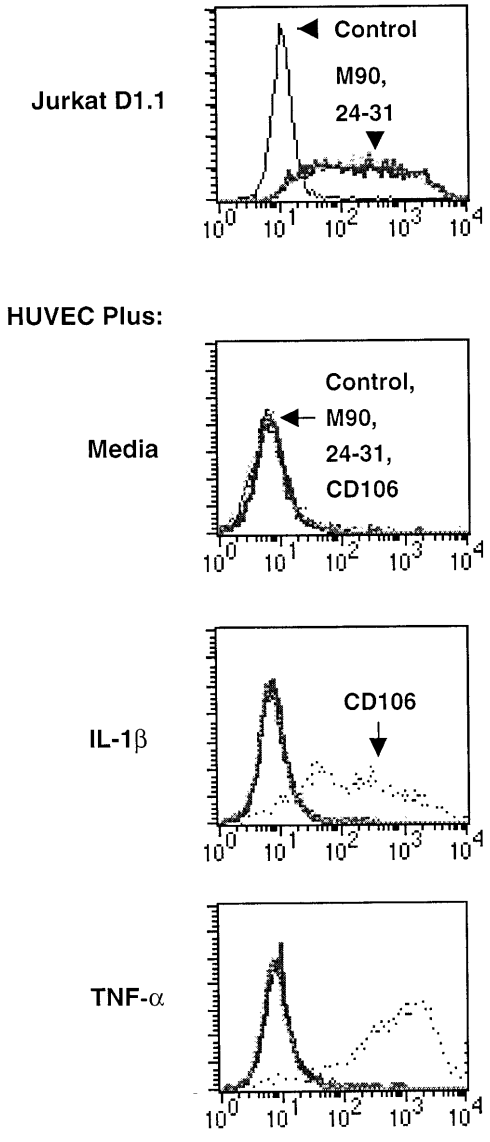

Fig. 3 Analysis of CD154 expression on resting or activated human umbilical vein endothelial cells in vitro. Overlapping histograms obtained from FACS analyses, demonstrating the lack of reactivity of anti-CD154 mAbs M90 or 24–31 with human umbilical vein endothelial cells (HUVEC). HUVEC were cultured for 24 h in the presence or absence of IL-1β or TNF-α. The cells were harvested by gentle scraping and FACS analyses performed. CD106 (VCAM-1) expression was utilized as a control for endothelial cell activation and $CD154^+$ D1.1 cells were used as a control for CD154 mAb reactivity. Identical results were obtained with anti-CD154 mAbs 5C8 and TRAP studying either resting or activated HUVEC (n=10) or saphenous vein endothelial cells (n=6)

tokine-activated endothelial cells, macrophages or smooth muscle cells express CD154 in vitro. Therefore, resting cells or cells activated with IFN-γ, TNF-α or IL-1β were analyzed for CD154 expression by flow cytometry. Jurkat D1.1 cells, which constitutively express CD154, were utilized as a positive control [10].

Utilizing four different mAbs we were unable to detect CD154 on resting or cytokine-activated HUVEC (Fig. 3) or SVEC (data not shown). Similar results were obtained with HUVEC isolated from 10 donors and SVEC obtained from 6 donors and cultured on either gelatin- or fibronectin-precoated wells. Moreover, CD154 was not detected on resting or cytokine-activated smooth muscle cells (Fig. 4) or monocytes (Fig. 5). All

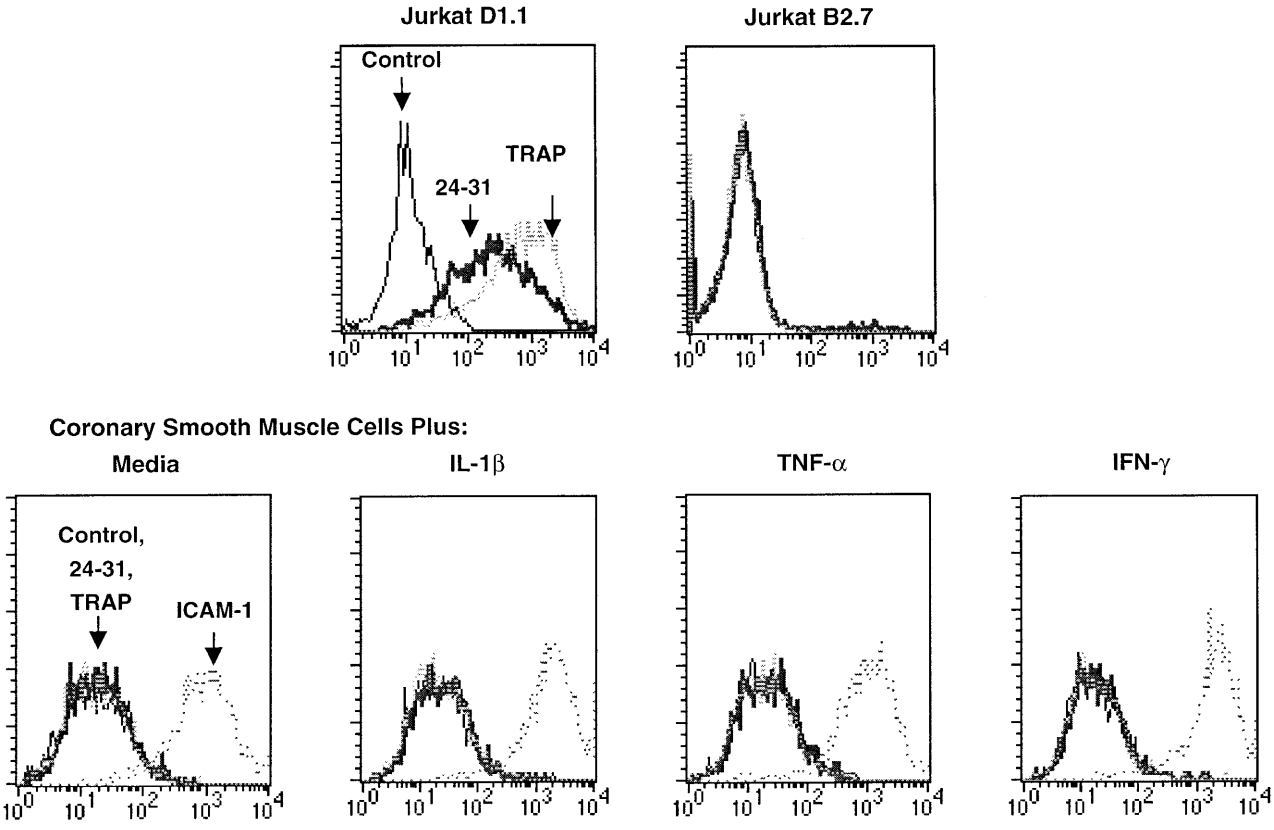

Fig. 4 Analysis of CD154 expression on resting or cytokine-activated human coronary artery smooth muscle cells in vitro. The *top panels* show overlapping FACS histograms demonstrating the reactivity of anti-CD154 mAbs 24–31 and TRAP with CD154$^+$ Jurkat D1.1 cells and CD154$^-$ Jurkat B2.7 cells. The *lower panels* show overlapping FACS histograms demonstrating the lack of reactivity of anti-CD154 mAbs TRAP or 24–31 with human coronary artery smooth muscle cells. The smooth muscle cells were cultured for 24 h in the presence or absence of IL-1β, TNF-α or IFN-γ, and harvested by gentle scraping, after which FACS analyses were performed. As a control, CD54 (ICAM-1) expression on the cells is also shown. Identical results were obtained with human aortic smooth muscle cells (n=3). Additionally, anti-CD154 mAbs 5C8 and M90 did not react with resting or cytokine-activated smooth muscle cells

anti-CD154 mAbs, but not isotype control mAbs, reacted with D1.1 cells (Fig. 3–5).

Immunohistochemical analysis of CD40 expression in CA and TCAD

CD40 immunoreactivity was up-regulated and widely distributed in the lesions of native CA (Fig. 6a). CD40 expression was noted on endothelial cells, smooth muscle cells, macrophages and "foam" cells. CD40-expressing cells accumulated in areas of inflammation and neovascularization, which were located at the shoulder of fibroatheromatous plaques. The mean number of CD40-positive cells was higher in intimal lesions of native CA than in control arteries (score 2.2±0.7 versus 0.5±0.6, P<0.05, Table 1). Dual immunostaining with macrophage- or smooth muscle cell-specific markers confirmed that these cells and "foam" cells expressed CD40 (Figs. 6c, d). Interestingly, CD40$^+$ smooth muscle cells were present in the intima near inflammatory infiltrates, whereas smooth muscle cells in the arterial media did not show positive immunoreactivity for CD40 (Fig. 6d). Analysis of serial sections stained with CD40 or the endothelial marker CD31 suggested that endothelial cells lining the intimal neovessels and adventitial vasa vasorum were also strongly CD40$^+$ (data not shown).

In arteries from patients with TCAD, the pattern of distribution of CD40 expression was similar to that seen in native CA. However, the average score for CD40 immunoreactivity was significantly higher (P<0.05, Kruskal-Wallis test) in TCAD (score 3.3±0.9) than in native CA or control arteries (Table 1). Double immunostaining again indicated that intimal foam cells (Fig. 6b) smooth muscle cells and macrophages expressed CD40 (Figs. 6c, d). Endothelial cells lining the vascular lumen, intimal neovessels and adventitial vasa vasorum were also strongly CD40$^+$. The degree of CD40 reactivity correlated significantly (P<0.05) with the amount of neovessels, macrophages and T cells in these lesions, with a Spearman correlation coefficient ranging from 0.79 to 0.85. Overall, lesions of TCAD showed a higher degree of luminal stenosis with smaller or absent lipid cores and more foam cells and consistently contained a higher number of CD154$^+$ and CD40$^+$ cells than the atheromatous plaques of CA.

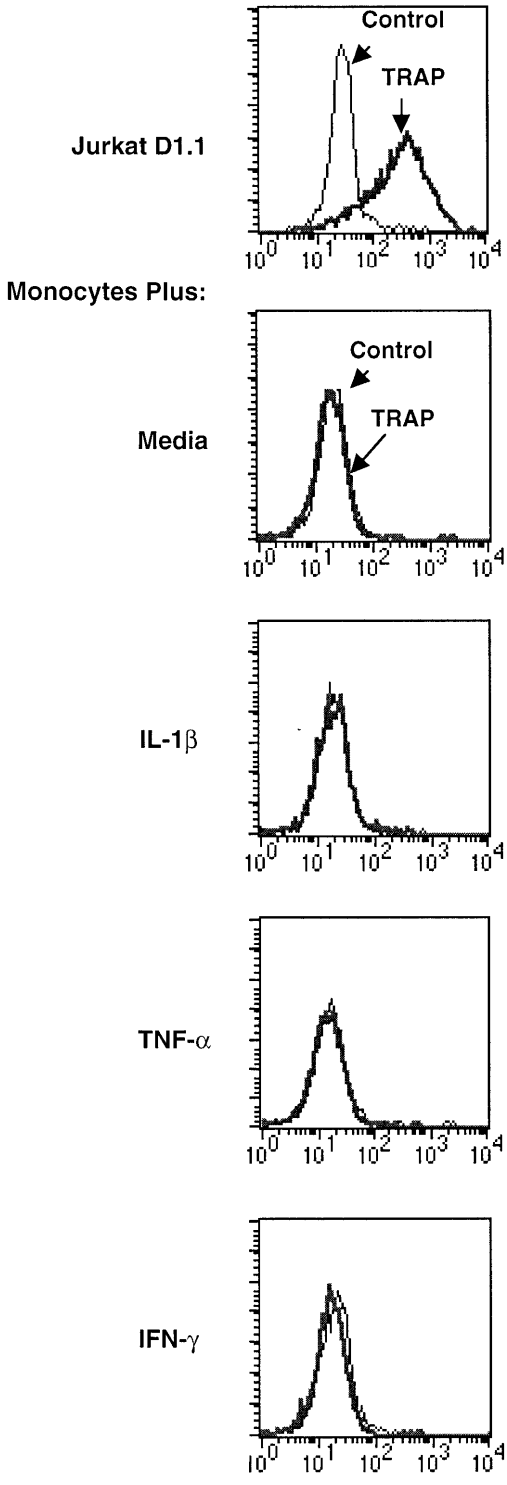

Fig. 5 Analysis of CD154 expression on resting or cytokine-activated human monoyctes in vitro. The *top panel* shows an overlapping FACS histogram demonstrating the reactivity of PE-conjugated anti-CD154 mAb (TRAP) with $CD154^+$ Jurkat D1.1 cells. The *lower panels* show overlapping FACS histograms demonstrating the lack of reactivity of PE-conjugated anti-CD154 mAb monocytes. Monocytes were cultured for 24 h in Teflon-coated containers in the presence or absence of IL-1β, TNF-α or IFN-γ prior to harvesting and analysis. This experiment was repeated 3 times with similar results. Additionally, resting or cytokine-activated monocytes cultured on plastic and harvested by scraping did not express CD154 (not shown)

Relationship between CD40 expression and CD54 (ICAM-1) and CD106 (VCAM-1) expression in CA and TCAD lesions

Macrophages and endothelial cells in CA and TCAD express intercellular adhesion molecules, such as CD54 and CD106, that regulate trafficking of leukocytes into lesions. Because ligation of CD40 induces up-regulation of CD54 and CD106 on cells in vitro, we examined whether CD40 was co-expressed with these intracellular adhesion molecules in atheromas. Dual immunolabeling demonstrated cellular co-localization of CD40 (stained brown) with both CD54 (stained red; Fig. 6e) and CD106 (stained red; Fig. 6f) in CA and TCAD lesions. For endothelial cells, co-localization of adhesion molecules and CD40 was preferentially noted in areas of neovascularization and, interestingly, in the vasa vasorum in both CA and TCAD. Scores for immunostaining of the adhesion molecules in TCAD were higher than in CA or normal coronary arteries (Table 1). There was a significant correlation ($P<0.05$) between CD40 scores and those for CD54 (ICAM-1; $r=0.85$, r=0.82) and CD106 (VCAM-1; $r=0.72$, $r=0.89$) in CA and TCAD, respectively

Discussion

TCAD and native CA are inflammatory diseases mediated by complex interactions between activated T cells, endothelial cells, macrophages and smooth muscle cells. CD154 is an activation-induced $CD4^+$ T cell surface molecule that delivers contact-dependent activating signals to $CD40^+$ target cells, including endothelial cells, macrophages and smooth muscle cells [5, 6, 12, 34]. Previous studies in mice strongly suggest that CD154-CD40 interactions contribute to the pathogenesis of TCAD and CA [9, 13]. Moreover, CD154 and CD40 are reported to be widely expressed in human carotid atherosclerotic lesions [12]. To investigate the possible role of CD154-CD40 interactions in mediating TCAD we studied the expression and cellular distribution of CD154 and CD40 in human TCAD lesions. Moreover, we examined CD154 and CD40 expression in human coronary artery atherosclerotic lesions. Our studies demonstrate that: (1) CD40 expression is markedly up-regulated on intimal and neovascular endothelial cells, as well as intimal foam cells, macrophages and smooth muscle cells in TCAD and CA; (2) CD154 expression is confined to infiltrating T cells in lesions of both diseases; (3) $CD40^+$ cells in CA and TCAD lesions co-express CD54 (ICAM-1) and CD106 (VCAM-1); and (4) normal coronary arteries do not contain CD154 expressing cells and CD40 immunoreactivity is restricted to luminal endothelial cells in normal vessels. These studies are the first to demonstrate infiltrating $CD154^+$ T cells and up-regulated CD40 expression in human TCAD lesions. In contrast to a previous report [12], our studies suggest that CD154 expression has a restricted cellular distribution in native atherosclerotic lesions.

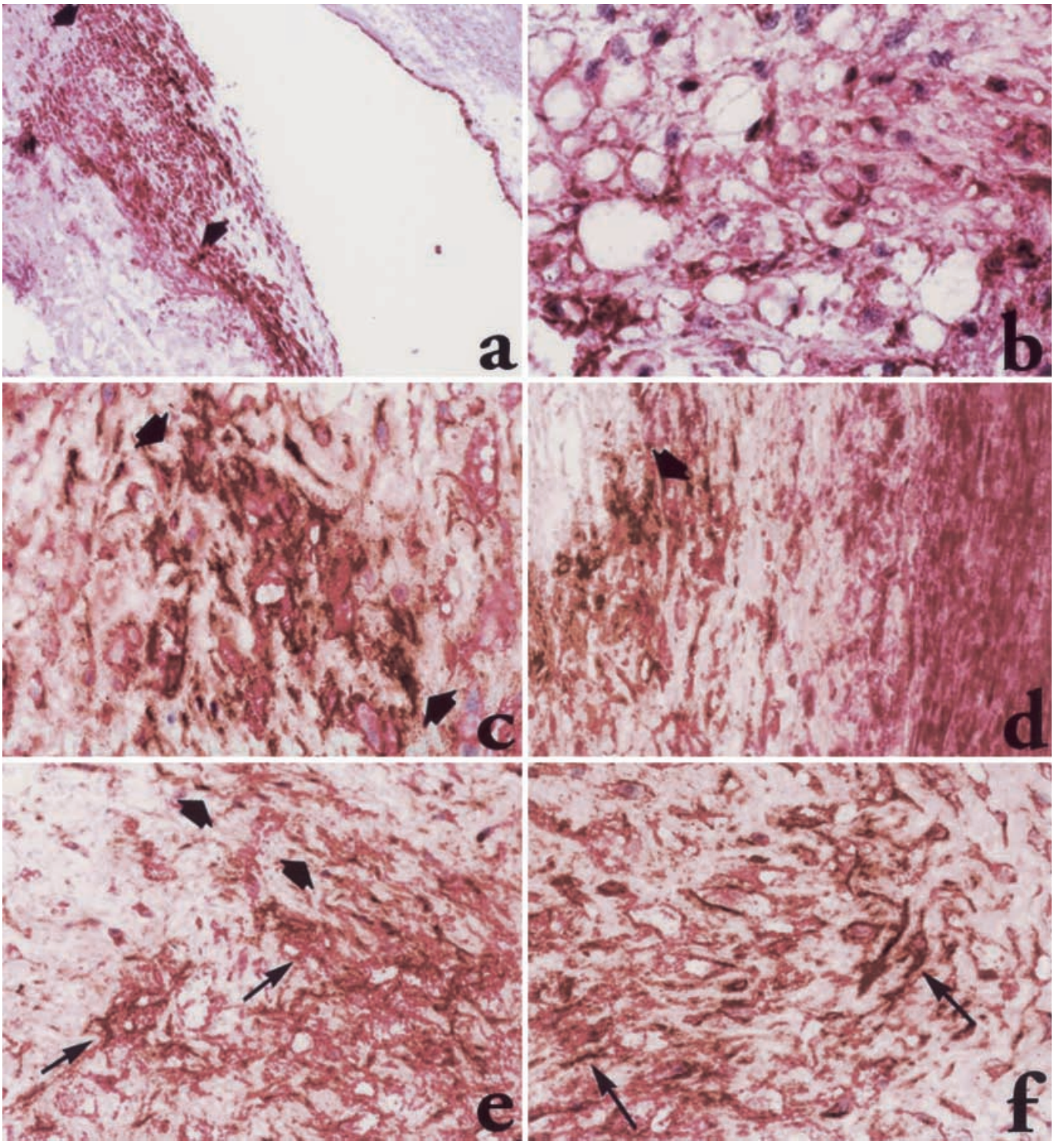

Fig. 6a-f Cellular distribution of CD40 expression and association with intercellular adhesion molecules in CA and TCAD. Shown are **a**, **e**, **f** intimal lesions of CA or **b**, **d** TCAD (Fig. 6b, d). **a**, **b** Intense CD40 expression and diffuse cellular distribution of CD40 expression is demonstrated in lesions; **b** is a high-power view of the area marked by the *arrows* in **a**. Dual immunolabeling for CD40 (*brown*) and CD68 or smooth muscle actin (*red*) indicates that lesional macrophages (**c**) and smooth muscle cells (**d**) express CD40. *Arrows* in **c** and **d** demonstrate representative $CD40^+$ macrophages or smooth muscle cells, respectively. Note that intimal smooth muscle cells in **d** are positive for CD40, whereas medial smooth muscle cells are CD40 negative, suggesting differences in states of cellular activation. Dual immunolabeling studies indicate that CD40 (*brown*) co-localizes on cells with **e** CD54 (*red*) and **f** CD106 (*red*). *Arrows* in **e** and **f** demonstrate representative cells co-expressing $CD40^+$ and CD54 or CD106, respectively. (Original magnification: **a** ×100, **b–f** ×400)

Staining serial sections with anti-CD4 mAb or anti-CD8 mAb strongly suggest that CD154$^+$ lymphocytes are CD4$^+$ T cells in lesions of both diseases. Similar results were obtained with four different anti-CD154 mAbs. CD154 immunoreactivity was usually dim and either cytoplasmic or cell surface associated. A similar pattern of CD154 immunoreactivity was noted in our previous study of CD154 and CD40 expression in glomerulonephritis [36]. The weak and frequent cytoplasmic staining pattern of CD154 expression in inflammatory tissues may be related to the transient nature of CD154 expression on activated T cells [10] and the fact that engagement of CD40 on target cells induces rapid down-modulation of CD154 by receptor-mediated endocytosis [33] and shedding [4]. These regulatory mechanisms probably serve to focus CD154-mediated signaling events on appropriate cognate target cells.

We found that CD40 expression was markedly up-regulated on many cells in the lesions of both diseases. This is in agreement with a prior report studying CD40 expression in carotid atherosclerosis lesions [12]. We noted that macrophages and "foam" cells expressing CD40 were particularly prominent in the inflammatory infiltrate of the "shoulder" regions of lipid-rich plaques, which are known to contain dense inflammatory infiltrates [30]. CD40 expression was also up-regulated on luminal endothelial cells in both diseases, and this was particularly prominent in TCAD. Intimal neovessel and adventitial vasa vasorum endothelial cells in both diseases were strongly CD40$^+$. CD40-expressing smooth muscle cells were present in the intima of both CA and TCAD, usually in close proximity to inflammatory infiltrates. Interestingly, smooth muscle cells in the media of the same vessels were CD40 negative. IFN-γ up-regulates CD40 expression on many cells in vitro [5, 34, 35], including smooth muscle cells [12], and this effect is enhanced by cytokines such as IL-1β and TNF-α [5] (also our unpublished observations). Therefore, the marked up-regulation of CD40 expression on many cell types in these lesions may be a consequence of cytokine release by lesional T cells, macrophages and other cells. Double immunostaining indicated that many CD40$^+$ cells also co-express intercellular adhesion molecules CD54 and CD106. TCAD lesions consistently contained higher numbers of lymphocytes and cells expressing CD40, CD154, and intercellular adhesion molecules than did CA lesions. TCAD lesions also demonstrated a greater degree of luminal stenosis and intimal fibroplasia. Hence, the increased level of CD154 and CD40 expression in TCAD lesions correlates with a more rapid course of vascular stenosis compared to CA lesions.

Mach et al. have presented evidence that CD154 expression is up-regulated on macrophages, smooth muscle cells and endothelial cells in carotid atherosclerosis lesions [12]. Moreover, this group demonstrated that macrophages, smooth muscle cells and endothelial cells constitutively expressed CD154 in vitro and cytokines further up-regulated CD154 expression on all these cell types [12]. Similarly, Reul found that cytokine activated human umbilical vein endothelial cells expressed CD154 [21]. However, in our studies of atherosclerosis, CD154 expression was restricted to T cells in TCAD and CA lesion. Specifically, utilizing four different anti-CD154 mAbs we did not observe in situ CD154 expression on macrophages, endothelial cells or smooth muscle cells in either disease. Moreover, in our hands, CD154 expression was not detected by FACS analysis on resting or cytokine-activated human umbilical vein endothelial cells, saphenous vein endothelial cells, macrophages or smooth muscle cells.

The reasons for these discrepancies are not entirely clear, but may be due in large part to the reagents utilized. In this regard, we found that the polyclonal anti-CD154 antibody (Santa Cruz Biotechnology) used by Mach et. al. [12] indeed stains a variety of cells, in addition to T cells, in situ in atherosclerotic lesions, as well as in human tonsil. Additionally, the reagent fails to bind CD154 expressing Jurkat T cells or CD154 transfectants in vitro by FACS analysis (our unpublished observations). As reported by Mach et al. [12], we found that the FITC conjugated anti-CD154 mAb purchased from Calbiochem (La Jolla, Calif.) reacts with many cell types in vitro, including a human Jurkat cell subclone (B2.7) that lacks CD154 mRNA or cell surface expression and has been well characterized as unable to activate CD40$^+$ target cells [10, 32, 34, 35]. The same anti-CD154 mAb in the unconjugated form, as well as three other anti-CD154 mAbs, do not react with resting or cytokine-stimulated smooth muscle cells, endothelial cells or macrophages when analyzed by FACS in vitro (Figs. 3–5) or when analyzed in atherosclerotic lesions by immunohistochemistry (Figs. 1, 2). Together, we interpret these data to suggest that the polyclonal anti-CD154 mAb and the Calbiochem FITC conjugated anti-CD154 mAb are cross-reacting with molecules other than CD154 and that the main cell type expressing CD154 in lesions are CD4$^+$ T cells. Additionally, a common morphologic feature of atherosclerotic lesions, as well as other immune-mediated inflammatory lesions, is the close apposition of endothelial cells and T cells. Therefore, T cells may be mistaken for lining endothelial cells, particularly in immunofluorescence studies (as utilized by Mach et al. [12]), where cellular morphology cannot be studied.

The models put forward for understanding the role of CD154–CD40 interactions in the pathogenesis of CA and TCAD will depend on the true cellular distribution of CD154 in lesions. The widespread cellular distribution of CD154 expression combined with co-localization on the same cell with CD40 in atheromas, as well as the constitutive expression of CD154 on endothelial cells, macrophages and smooth muscle cells in vitro, as suggested by Libby and colleagues [12], contradicts current concepts of CD154 expression and regulation of CD154–CD40 interactions. Our studies suggest that T cells enter lesions and express CD154 following antigen presentation and activation. In turn, the T cell would deliver CD154-dependent activating signals to the CD40$^+$ antigen-presenting cell and possible other CD40$^+$ target

cells. This hypothesis is supported by a recent study in mice demonstrating key roles of CD154–CD40 interactions in the propagation of pre-existing atheromas albeit with little effect on the initiation of lesions [11].

Mechanistically, this hypothesis is consistent with our finding that many $CD40^+$ cells in both CA and TCAD lesions co-express CD54 and CD106. We and others have demonstrated that ligation of CD40 up-regulates these adhesion molecules on $CD40^+$ cells in vitro [5, 6, 34]. CD154–CD40 interactions may participate in the pathogenesis of CA and/or TCAD via other mechanisms as well. For example, CD154-mediated signals also induce endothelial cells to secrete IL-6 and IL-8 [12] and promote a pro-coagulant surface by up-regulating tissue factor and down-regulating thrombomodulin expression [15]. Ligation of CD40 on macrophages also induces secretion of matrix metalloproteinases, as well as up-regulating iNOS and NO production [14, 29]. Interestingly, blockade of CD154–CD40 interactions in murine models of TCAD is associated with down-regulation of iNOS expression and reduction of TCAD lesions [9].

In summary, our studies are the first to demonstrate that CD154 is expressed on, and restricted to, T cells infiltrating TCAD lesions. The presence of $CD40^+$ target cells in the same lesions suggests that CD154–CD40 interactions may contribute to the immune-mediated inflammatory response characteristic of the disease. Our studies also demonstrate an identical pattern of CD154 and CD40 distribution in native CA lesions, supporting the idea that CA is at least partly a T-cell-driven chronic inflammatory process.

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