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Accessory molecules utilized by endothelial cells for allogeneic stimulation of T-lymphocytes

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Introduction

One of the major problems in allograft transplantation is rejection of the graft, as a result of an immune response mounted by the recipient against alloantigens expressed by the graft [1,2]. Owing to their unique anatomical position, endothelial cells (ECs) are among the first cells of the allograft that are encountered by the recipient's immune system. MHC class-I and class-II antigens expressed by the ECs may activate alloreactive CD8⁺ and CD4⁺ T-lymphocyte subsets respectively [3–10]. T-cell activation requires not only engagement of the T-cell receptor–CD3 complex, but also one or more co-stimulatory or accessory signals [11–16]. In several *in vitro* systems, the potent antigen-presenting and co-stimulatory capacities of ECs have been demonstrated [17–19]. In this study we have analysed the accessory signals involved in the proliferation of alloreactive peripheral blood T-lymphocytes induced by allogeneic MHC class-II molecules *in vitro*. Allogeneic MHC class-II molecules induced by treatment with interferon γ (IFN- γ) were presented by either ECs or fibroblasts (FBs). The expression of co-stimulatory molecules on both these cell types was compared. The T-cell-proliferation-inhibiting effect of a panel of antibodies directed against co-stimulatory molecules expressed by the ECs or against their counterstructures on the T-cells was assessed. Distinct inhibition of T-cell proliferation was observed with monoclonal antibodies (mAbs) directed against CD2, lymphocyte-function-associated antigen (LFA)-3, LFA-1 or a combination of anti-[intercellular adhesion molecules (ICAM)-1 and -2]. We conclude that CD2–LFA-3 and LFA-1–ICAM interactions are crucially involved in allogeneic T-cell–EC interactions.

Abbreviations used: EC, endothelial cell; IFN- γ ; interferon γ ; FB, fibroblast; mAb, monoclonal antibody; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule; LFA, lymphocyte-function-associated antigen.

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Materials and methods

ECs and FBs

ECs and FBs were isolated from human umbilical cord vein and foreskin fragments respectively as described previously [20]. Cells were serially subcultured under standard conditions. Passages 3 to 15 were used; no influence of the passage number on the outcome of the experiments was observed.

Monocytes and T-cells

Monocytes and T-cells were isolated as described previously [20–22]. Briefly, leucocyte-enriched cell suspensions were obtained from healthy volunteers by cytopheresis. Mononuclear cells were isolated from these suspensions by centrifugation on Ficoll (density 1.077 g/ml; Pharmacia, Uppsala, Sweden), and monocytes and lymphocytes were subsequently separated by counterflow centrifugation. T-cells were isolated from the lymphocyte fraction by rosetting with 2-aminoethylisothiuronium bromide-treated sheep red blood cells. Monocytes as well as T-cells were cryopreserved in liquid nitrogen until use.

Allogeneic T-cell-proliferation assay

T-cells were cultured in 96-well U-bottomed microtitre plates. Per incubation, 1.5×10^5 T-cells and 5.0×10^4 ECs or FBs were added. ECs and FBs were γ -irradiated with 4000 rad before addition. In most experiments, HLA class-II expression was induced on ECs or FBs before use by a 3-day incubation with 200 units/ml IFN- γ (a gift from Boehringer-Ingelheim, Ingelheim, Germany). After 5 days of culture, T-cell proliferation was measured as [³H]thymidine incorporation.

Antibodies

mAbs used for inhibition experiments and/or immunofluorescence are listed in [20]. mAbs directed against adhesion molecules were selected for their capacity to block interactions between the adhesion molecule and its ligand. In inhibition experiments, mAbs were present during the entire duration of the EC–T-cell co-culture.

Immunofluorescence

ECs or FBs were detached from culture flasks non-enzymically. Cells were washed and incubated with mAb at 0°C in suspension, stained with FITC-conjugated sheep anti-mouse antibody, and analysed using a flow cytometer.

Results

Analysis of antigen expression on human ECs and FBs before and after treatment with IFN- γ

Expression of MHC class-I and class-II antigens and of co-stimulatory molecules was determined by immunofluorescence followed by flow-cytometry analysis. Both ECs and FBs expressed MHC class-I antigens constitutively, and did not express class-II antigens. Treatment with IFN- γ increased class-I antigen expression, especially on ECs, and induced a marked class-II antigen expression on both ECs and FBs (Figure 1). The expression levels of HLA class-I and -II molecules on IFN- γ -treated FBs were somewhat lower than, but comparable with, those on IFN- γ -treated ECs. The expression profiles of co-stimulatory molecules on ECs and FBs were very similar. LFA-3 had a low expression level on

both ECs and FBs which was hardly affected by treatment with IFN- γ . Vascular cell adhesion molecule (VCAM)-1 and E-selectin were absent from all the cells tested, as was the B7/BB1 molecule (results not shown). ICAM-1 was expressed on a subpopulation of untreated ECs and FBs, and was considerably up-regulated by treatment with IFN- γ . ICAM-2 was constitutively expressed on ECs but not on FBs, and was not affected by treatment with IFN- γ (results not shown).

Allogeneic HLA class-II antigen expression on ECs is necessary, but not sufficient for induction of T-cell proliferation

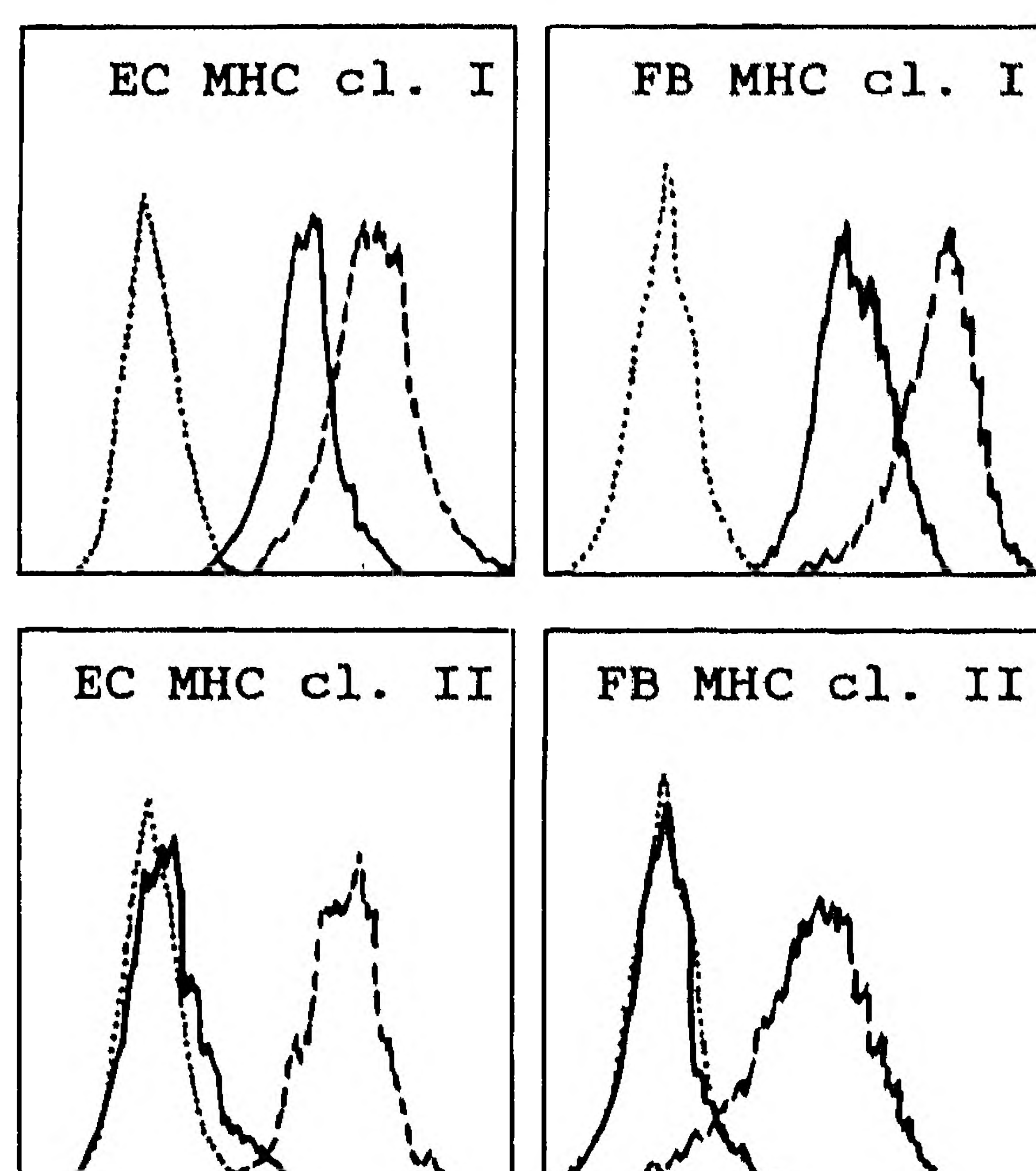
Both untreated and IFN- γ -treated ECs and FBs were cultured in the presence of purified allogeneic peripheral blood T-cells. T-cell proliferation was determined for different combinations of T-cell, FB and EC donors. A representative experiment is shown in Figure 2. Untreated ECs induced only marginal T-cells proliferation, ranging in these experiments between a 3- and 38-fold increase in [³H]thymidine uptake as compared with T-cells alone. ECs treated with IFN- γ , however, induced a 22- to 257-fold increased T-cell proliferation. In contrast,

Figure 1

Analysis of MHC class-I (top) and class-II (bottom) expression on ECs (left) and FBs (right) as determined by indirect immunofluorescence followed by FACS analysis

....., Cells stained with negative control antibody; ———, untreated cells stained with anti-MHC antibodies; - - - - - , IFN- γ -treated cells (3 days; 200 units/ml) stained with anti-MHC antibodies.

cell number \uparrow

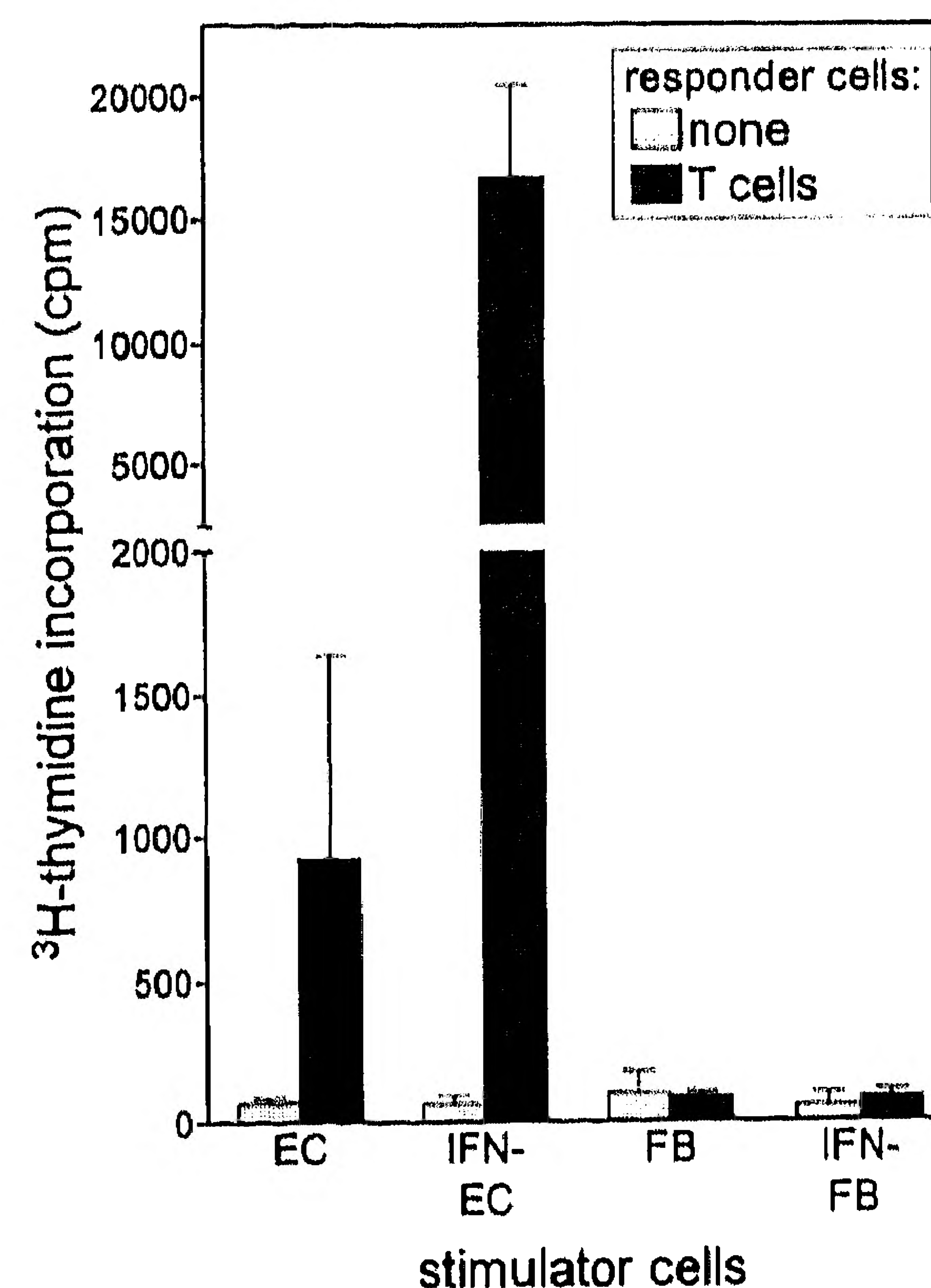


log mean fluorescence intensity \rightarrow

Figure 2

Proliferation of purified peripheral blood T-lymphocytes stimulated with untreated ECs and FBs, or with IFN- γ -treated (3 days; 200 units/ml) ECs or FB (IFN-EC and IFN-FB respectively)

T-cell proliferation is expressed as [³H]thymidine incorporated (c.p.m.).



neither untreated nor IFN- γ -treated FBS were able to induce T-cell proliferation to significant levels. Considerable differences existed between the levels of T-cell proliferation within the different T-cell/EC combinations, probably reflecting the degree of incompatibility between stimulator ECs and responder T-cells. To establish the involvement of MHC class-II molecules in the allogeneic stimulation of T-cell growth more directly, we added mAb directed against MHC class-II molecules or against CD4 to co-cultures of purified T-cells and IFN- γ -treated ECs, and tested their influence on T-cell proliferation. Anti-(MHC class-II) and anti-CD4 mAbs inhibited T-cell proliferation by 65 and 80% respectively (results not shown), establishing the importance of these molecules in the T-cell activation process.

Involvement of EC-associated adhesion molecules in the allogeneic response

To investigate the importance of co-stimulatory molecules in the generation of an allogeneic

T-cell response, we added mAb directed against a number of these molecules to co-cultures of T-cells and IFN- γ -treated ECs. The maximal inhibition level we could induce with control mAb (directed against antigens on either ECs or T-cells not involved in the activation process) was 25%. We therefore considered any mAb that induced 25% or less inhibition of T-cell proliferation as non-inhibitory. The inhibitory effects we observed are summarized in Table 1. Antibodies directed against B7 (the counterstructure of T-cell activation molecule CD28 [23-25]; inhibition was also assessed by incubation with a fusion protein of Ig and CTLA-4, like CD28 a counterstructure of B7, with the same result), CD26 (dipeptidylpeptidase IV, a molecule with co-stimulatory properties expressed by T-cells [26]) and CD31 (an adhesion molecule expressed by both ECs and a subset of T-cells [27]) were not inhibitory. Since previous publications by Savage et al. [28] and Hughes et al. [29] suggested a role for the CD44 molecule in EC-supported allogeneic T-cell proliferation, we

Summary of T-cell proliferation by (combination of) antibodies directed against co-stimulatory molecules

| Inhibition | Antibodies |
|------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| No inhibition (<25%) | B7*, CD26, CD31, E-selectin, Endoglin, CD44 (3) [†] , ICAM-1, CD44 (1) [†] , ICAM-2, ICAM-1 + ICAM-2, LFA-1 α -chain, LFA-1 β -chain, LFA-1 + ICAM-1 + ICAM-2, CD2 (1) [‡] , CD2 + LFA-3, VLA-4 α -chain, VLA-4 + VCAM-1 |
| Moderate inhibition (25-75%) | ICAM-1/2 + LFA-3 + VCAM-1, LFA-1 + CD2 + VLA-4 |
| Strong inhibition (>75%) | *Inhibition studies performed with anti-B7 mAb and CTLA-4-Ig fusion protein. [†] Of four anti-CD44 mAbs tested, three were non-inhibitory, one was moderately inhibitory. [‡] Of two anti-CD2 mAbs tested, one was moderately inhibitory, and one was strongly inhibitory. |

Table 1

tested four different anti-CD44 mAbs in our model system. A modest inhibition (35%) could be observed with mAb 212.3, but not with the other three anti-CD44 mAbs. Whereas anti-LFA-1 mAbs were distinctly inhibitory (60–70%), anti-ICAM-1 inhibited no more than the control mAb. A somewhat higher inhibition was observed with anti-ICAM-2, whereas the combination of anti-ICAM-1 and -2 inhibited in an additive manner. A cocktail of mAbs directed against all components of the LFA-1/ICAM adhesion couple, however, was no more inhibitory than the anti-LFA-1 mAb alone.

Two mAbs directed against CD2 were inhibitory up to 55–85%. This inhibition was, however, only partially mirrored by the inhibition obtained with mAbs directed against the endothelial counterpart, LFA-3. mAbs directed against two alternative counterstructures of CD2, CD48 and CD59, were not inhibitory. Of the third co-stimulatory couple investigated, VLA-4/VCAM-1, only the anti-VLA- β mAb was moderately inhibitory. Almost complete blockage of T-cell proliferation could be observed with a cocktail of all mAbs directed against adhesion molecules on the T-cell and, to a lesser extent, by blocking adhesion molecules on the endothelial cells.

Discussion

Numerous aspects of EC-mediated activation of T-cells have been extensively investigated in a number of previous studies (reviewed in [17–19]). Two types of interaction have to be distinguished: those occurring between autologous cells and those between allogeneic or xenogeneic cells. The autologous interactions have been the focus of attention during the past few years. Several studies have revealed the importance of interactions between CD2 and LFA-3 molecules as a source of EC-derived co-stimulatory activity [22,28–30]. In the allogeneic situation, the interactions between lymphocytes and the endothelium are more complex. *In vivo*, they occur in vascularized allografts, where recipient lymphocytes enter the graft via the bloodstream, and encounter donor ECs. Via adhesion events probably similar to or identical with those taking place in inflammatory lymphocyte–vessel wall interactions, binding to the endothelium will be established, followed by extravasation into the allogeneic tissue. In addition to the molecular interactions that occur in the autologous situation, MHC class-I and class-

II antigens expressed by the vascular endothelium may deliver initial activation and proliferation signals to recipient T-lymphocytes. The notion of T-cell activation by allogeneic ECs is supported by a large body of experimental evidence [7,10,31–34]. In this study we investigated the role of co-stimulatory molecules in the induction of allogeneic T-cell activation, using an *in vitro* co-culture system with human umbilical vein ECs and purified peripheral blood T-lymphocytes.

Human FBs, induced by IFN- γ to express MHC class II molecules, were unable to induce a T-cell response, confirming previously published data [35–37]. We therefore conclude that accessory signals, provided by ECs, play a crucial additional role. We assessed the expression of ICAM-1 and -2, VCAM-1, E-selectin, CD31, B7 and LFA-3 on untreated and IFN- γ -treated ECs and FBs, and found that levels of expression on the two cell types were comparable, with the exception of ICAM-2. This might suggest that this alternative ligand for LFA-1 may have a specific function in the accessory function of ECs. Antibodies directed against ICAM-2 were indeed more inhibitory than anti-ICAM-1 mAbs in our T-cell-proliferation assay, but did not inhibit T-cell proliferation completely, suggesting the existence of one or more additional, as yet unidentified, EC-specific factors. Our inhibition experiments further showed involvement of CD2/LFA-3, but not of VLA-4/VCAM-1, B7/CD28/CTLA-4, E-selectin, CD31/CD31 or CD26 interactions in T-cell proliferation induced by allogeneic endothelial cells, confirming data presented in several recent publications [31,32,37–40]. In the case of VCAM-1, E-selectin and B7, this is not surprising, since these molecules are not expressed by either unstimulated or IFN- γ -treated ECs. Taken together, our inhibition results indicate that both LFA-1–ICAM-1 and -2, CD2–LFA-3 interactions play a role in allogeneic stimulation of T-cells by ECs. Similar observations to those made in this report were also made in a previous study, in which we examined the accessory capacities of ECs in T-cell proliferation induced by anti-CD3 mAb [22].

Recently some progress has been made in investigating the effects *in vivo* of reagents that block adhesion events involved in EC-mediated T-cell activation. Administration of a cocktail of anti-LFA-1 and anti-ICAM-1 mAbs could prolong indefinitely the survival of cardiac allografts

between fully incompatible mouse strains [41]. Thurlow et al. [42] did not find any effect of anti-CD2 mAb administration on renal allograft rejections by humans, but cocktails of anti-CD2 and anti-LFA-3 mAbs may have different effects. Recently, increased survival of cardiac allografts in a primate model achieved by administration of an LFA-3-Ig chimaeric molecule was reported by Kaplon et al. [43]. Further studies on the role of adhesion molecules in allograft rejection in experimental animals and in man may provide alternative strategies for rejection therapy besides the conventional immunosuppressive treatments aimed at the depletion of immunocompetent T-cells.

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Non-conventional mechanisms of T-cell co-stimulation by endothelial cells

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Introduction

Lymphocyte–endothelial interactions are bidirectional, complex and dynamic. Contact with T-cells can change endothelial behaviour [1], and contact with endothelial cells can change T-cell behaviour (reviewed in [2]). Studies in the latter area have focused primarily on the ability of endothelial cells to serve as alloantigen-presenting cells (APCs) for T-cells. This may have obscured other more subtle effects of endothelial cells on T-cell behaviour. We have developed gonadal vein endothelial cell (GVEC) lines from cadaver organ donors that can be obtained along with matched autologous splenocytes [3]. This permits an examination of non-allogeneic lymphocyte–endothelial interactions. In this experimental system, we have observed a non-conventional mechanism of T-cell co-stimulation that involves interleukin (IL)-2 and endothelial cell contact.

Methods

Cell populations

Peripheral blood mononuclear cells were obtained from fresh heparinized blood, collected by venipuncture from normal individuals, isolated using Ficoll–Hypaque gradients. Cells recovered from the peripheral blood mononuclear cell interface were washed three times in Seligman's balanced salt solution (Gibco) and resuspended in complete Dulbecco's modified Eagle's medium [4].

CD3⁺ T-cells were isolated from peripheral blood mononuclear cells by negative selection, with a commercially available cocktail of monoclonal antibodies (mAbs) and C' (T Lympho-Kwik; One Lambda Inc., Canoga Park, CA, U.S.A.) using the methods of Clouse et al. [5]. In order to remove any remaining APCs, the cells were added to a 250 cm² culture flask and incubated with the flask on its side at 37°C under 10% CO₂ for 45 min. Non-adherent cells were collected, washed once, and adjusted to 1 × 10⁶/ml, and kept at 4°C until use. Cells prepared in this manner routinely stained >95% positive for CD3 by cytofluorimetric analysis, with undetectable levels of monocyte contamination (as verified by the absence of cells staining for CD33 or CD14 and the inability to proliferate in response to phytohaemagglutinin).

CD4⁺ T-cells were isolated from first-cycle T-Lympho-kwik-purified CD3⁺ cells by treatment with Helper T-Lympho-kwik (One Lambda). Cells prepared in this manner routinely stained >90% positive for CD4 by cytofluorimetry, with <2% contamination by CD8⁺ cells and undetectable levels of monocyte contamination (as verified above).

GVECs were isolated from fresh gonadal vein obtained from cadaveric donors and propagated as previously described [3]. Cultures were expanded in endothelial cell growth medium, consisting of M199 medium (Gibco) supplemented with 20% (v/v) heat-inactivated fetal bovine serum (UBI, Lake Placid, NY, U.S.A.), 100 µg/ml endothelial growth factor [6], 12 units/ml sodium heparin and 20 mM HEPES buffer. Before use, all endothelial cell lines were characterized by flow cytometry for the surface

Abbreviations used: APC, alloantigen-presenting cell; GVEC, gonadal vein endothelial cell; IL, interleukin, mAb, monoclonal antibody; TNF-α, tumour necrosis factor α; sTNFR, soluble TNF receptor.