

Major Histocompatibility Complex Class II-expressing Endothelial Cells Induce Allospecific Nonresponsiveness in Naive T Cells

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Summary

The role of endothelial cells (EC) in initiating a primary T cell response is of importance in clinical transplantation and autoimmunity since EC are the first allogeneic target encountered by the recipient's immune system and may display tissue-specific autoantigens in the context of an inflammatory response. In this study, we have investigated the antigen-presenting cell function of human umbilical vein-derived EC (HUVEC), depleted of constitutively major histocompatibility complex class II⁺ cells and induced to express class II molecules by interferon- γ . The results show that HUVEC do not express B7 but can support proliferation by antigen-specific T cell clones. In contrast, they were unable to initiate a primary alloresponse using three independent HUVEC cultures and MHC class II-mismatched CD4⁺ T cells from eight donors. The response to HUVEC was reconstituted by *trans*-costimulation provided by DAP.3 transfectants expressing human B7.1. Coculture of peripheral blood T cells with EC expressing allogeneic DR molecules had markedly different effects on CD45RO⁺ and RA⁺ subsets. Subsequent reactivity of the RO⁺ T cells was unaffected by exposure to EC, indicating a neutral encounter. In contrast, culture with DR⁺ EC induced allospecific nonresponsiveness in RA⁺ T cells.

Induction of proliferation by naive CD4⁺ T cells involves the delivery by the APC of at least two independent signals. The first signal results from the engagement of the TCR/CD3/CD4 complex, and the second from the interaction of CD28/CTLA-4 with the B7 family of costimulatory molecules expressed by the APC. The cells that are most efficient in providing both signals are the so-called "professional" APC. Preeminent among these are dendritic cells; macrophages and activated B cells may also be able to provoke primary T cell responses under some circumstances. Activation of secondary T cell responses has also been shown to require B7-mediated costimulation, although this appears not to apply to all T cells (Hargreaves, R.E.G. and Lechler, R.I., manuscript in preparation). Furthermore, antigen presentation by APC that cannot provide costimulation has been observed to render the Ag-specific T cells refractory to a subsequent challenge, as far as IL-2 production is concerned (1-3). This type of "nonprofessional" APC is represented by cells on which expression of MHC class II molecules can be induced by IFN- γ , such as keratinocytes (4, 5), myoblasts (6), and thyroid follicular cells (Lombardi, G., unpublished observation). Similar results have been achieved using cells in which expression of class II molecules has been engineered by transfection or by transgenesis (7, 8). In

this context, the consequences of antigen presentation by endothelial cells (EC)¹, is open to question.

The properties of EC as APC are likely to be important during local inflammatory responses and after transplantation of vascularized tissues. EC are the first alloantigen-expressing cells encountered by the recipient's immune system after transplantation, and human capillary EC have been shown to express MHC class II molecules in the heart, kidney, and liver (9-11). Also, because of their widespread distribution throughout the body, EC are continuously encountered by circulating T cells. If EC are able to induce primary T cell responses, this could create problems in the regulation of autoimmune reactivity. The capacity of EC to reactivate previously activated T cells is also of relevance to the effects of T cell *trans*-migration through EC monolayers at the sites of local inflammation.

The conclusions from previous studies suggest that EC can support mitogen-induced T cell proliferation (12), an-

¹Abbreviations used in this paper: B-LCL, EBV-transformed B-lymphoblastoid cell lines; EC, endothelial cells; HA, hemagglutinin; HLA, human leukocyte antigen; HS, human serum; HUVEC, human umbilical vein-derived EC; TdR, thymidine.

tigen-specific T cell proliferation (13), and primary alloresponses (14–16).

In this study, we have investigated the APC function of human EC, depleted of conventional APC, and induced to express MHC class II molecules by treatment with IFN- γ . The results obtained show that human EC lack the expression of B7 family of molecules, but can support proliferation of T cell clones when costimulation mediated by B7 molecules is not required. On the contrary, they were unable to initiate a primary alloresponse by resting CD4⁺ T cells. Furthermore, recognition of alloantigen on EC by the CD45 RA⁺ subpopulation of peripheral blood T cells induced a state of allospecific unresponsiveness.

Materials and Methods

Antigens and Mitogens. The synthetic hemagglutinin (HA) peptide HA 307-319 was synthesized by the Imperial Cancer Research Fund Peptide Unit (London) and kindly provided by Dr. H. Stauss (ICRF, London, U.K.). PHA was purchased from Sigma Chemical Company Ltd. (Poole, Dorset, UK).

mAbs. The following mAbs were used for staining cells for flow cytometric analysis as hybridoma supernatants: anti-B7/BB1 (anti-B7.1; Becton Dickinson, Cowley, Oxford, UK), BB1 (anti-B7.1; kindly provided by J. Ledbetter, Bristol-Myers Squibb, Seattle, WA) (17), and L243 (anti-HLA DR α ; American Type Culture Collection [ATCC], Rockville, MD). The following Abs were used in purified form for preparation of human umbilical vein-derived ECs (HUVECs) and CD4⁺ T cells: Leu 19 (anti-CD56; Becton Dickinson), mouse anti-human Ig (Fab specific; Sigma), OKT8 (anti-human CD8; ATCC), and L243. The OKT8 and L243 mAbs were purified from the culture supernatant on protein A-Sepharose beads by standard methods. Eluted antibody was dialyzed against three changes of PBS.

The fusion protein CTLA-4-Ig obtained from supernatant of COS transfectants (gift from P. Lane, Basel Institute for Immunology, Basel, Switzerland) (18), and the mAb TS 2.9 (anti-human LFA-3; ATCC), anti-DP (B7.21; ATCC), and anti-DQ (L2, 19) were purified as described above. The mAbs anti-CD45RO (UCHL1; gift from P. Beverley, ICRF) (20), and anti-CD45RA (SN 130; gift from G. Janossy, Royal Free Hospital, London) (21), were purified as described above and used for isolation of CD4 T cell subsets.

Separation and Culture of HUVECs. HUVEC were isolated from human umbilical cord veins by collagenase (Sigma) treatment according to a modification of the technique described by Jaffe et al. (22), and were depleted of contaminating MHC II⁺ cells using the Dynabead technique (DynaL Ltd., Merseyside, UK) according to the manufacturer's instructions.

Recovered cells were serially subcultured at 37°C with 5% CO₂ in Medium 199 (Sigma) supplemented with 20% heat-inactivated FCS, 2 mM glutamine (Flow Laboratories, Irvine, UK), 150 μ g/ml Endothelial Cell Growth Supplement (Sigma), 12 U/ml heparin (Sigma), 100 U/ml penicillin (Flow), 100 μ g/ml streptomycin (Flow), and 2.5 μ g/ml Fungizone (ICN Biomedicals Inc., Costa Mesa, CA) in tissue culture flasks (Greiner Labortechnik Ltd., Dursley, UK) coated with gelatin (Sigma).

At confluence, HUVEC were detached from the culture flasks using a solution of 0.125% trypsin in 0.2% EDTA (Life Technologies Ltd., Paisley, UK) and passaged.

For functional assays, HUVEC were used in the assays at passage 4–10.

Cell Lines. The DAP.3-B7 cell line was generated by transfecting murine DAP.3 cells with a cDNA clone encoding human B7.1 in the pcExV-3 vector (a kind gift from Dr. M. Jenkins, University of Minnesota, Minneapolis, MN) with pHyg, which contains a hygromycin B-resistance gene at ratios of 100:1, 50:1, and 20:1. Cells expressing the transfected gene were selected in medium containing 200 μ g/ml hygromycin B (Sigma). The fibroblast line was cultured in DMEM tissue culture medium (Flow Laboratories, Irvine, Ayrshire, Scotland) supplemented with 10% FCS, 2 mM glutamine, 50 IU/ml penicillin, and 50 μ g/ml streptomycin.

EBV-transformed B-lymphoblastoid cell lines (B-LCL), from the 10th International Histocompatibility Workshop (Princeton, NJ and New York, November, 1987), were cultured in RPMI 1640 tissue culture medium (Flow) supplemented with 10% FCS, 2 mM glutamine, 50 IU/ml penicillin, and 50 μ g/ml streptomycin.

Purification of CD4⁺ T Cells. PBMC were obtained by Ficoll-Hypaque (Pharmacia) centrifugation of heparinized blood, washed twice, and resuspended in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, 50 IU/ml penicillin, and 50 μ g/ml streptomycin. The cell preparation was then depleted of adherent cells by two 45-min rounds of adherence to plastic on tissue culture dishes at 37°C. The nonadherent cells were subsequently collected and incubated with a cocktail of purified mAbs (L243, OKT8, Leu19, and mouse anti-human Ig) at saturating condition for 30 min at 4°C. The cells were then washed twice to remove excess antibody and further enriched by magnetic immunodepletion. Briefly, mAb-treated cells were incubated with magnetic microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) coated with sheep anti-mouse Ig for 15 min at 4°C, and bead/mAb-coated cells were removed by passage through a magnetic column (miniMAC system; Miltenyi). The purified cells were resuspended in medium ready for the proliferation assay, and accessory cell contamination assessed by culture with 2 μ g/ml PHA in a 48-h assay.

T Cell Clones. Clones 7P.13, 7P.26, 7P.62, 7P.66, and 7P.69, specific for HA 307-319, were derived from PBMC isolated from a DRB1*0701 individual as described previously (5, 6). The clones were maintained in culture by weekly stimulation with autologous PBMC, HA peptide, and rIL-2 (Boehringer Mannheim Biochemicals, Mannheim, Germany) in RPMI 1640 medium supplemented with 10% human serum (HS), 2 mM glutamine, 50 IU/ml penicillin, and 50 μ g/ml streptomycin. For use in experiments, the T cells were purified by isolation on a Ficoll-Paque gradient 7 d after restimulation and washed five times by low speed centrifugation (210 g for 5 min) before use.

Flow Cytofluorimetric Analysis. For flow cytofluorimetric analysis, HUVEC that had been previously detached by trypsin-EDTA treatment were fixed with 4% paraformaldehyde in PBS for 30 min at 4°C, washed, and permeabilized with 0.1% saponin in PBS for 30 min at 4°C. The cells were subsequently stained with the indicated mAbs for 30 min at 4°C. After two further washes in PBS with 2% FCS, the cells were incubated for 30 min at 4°C with 100 μ l of 1:50 dilution of fluoresceinated sheep anti-mouse Ig (Amersham International, Amersham, UK). After two additional washes, stained cells were analyzed using the EPICS Profile flow cytometer (Coulter Electronics, Luton, UK).

For surface molecule staining (L243, B7, BB1, and CTLA-4-Ig), fixation and permeabilization steps were not performed.

T Cell Proliferation Assays. T cell clones (10⁴ cells/well) were cultured in the presence of HUVEC (treated with 40 Gy X irradiation) or B-LCL (2 \times 10⁴ cells/well, treated with 120 Gy X irradiation) prepulsed with HA peptide for 16 h in flat-bottomed

microtiter plates (Flow) in a total volume of 200 μ l in RPMI 1640 medium supplemented with 10% HS, 2 mM glutamine, 50 IU/ml penicillin, and 50 μ g/ml streptomycin.

After 48 h, wells were pulsed with 1 μ Ci 3 H-Thymidine (TdR) (Amersham), and harvested onto glass fiber filters 18 h later. Proliferation was measured as 3 H-TdR incorporation by liquid scintillation spectroscopy. The results are expressed as the mean of triplicate cultures. SEs were routinely <10%.

For primary MLR assays, purified human CD4⁺ T cells (10⁵ cells/well) were cultured with different numbers of either irradiated allogeneic HUVEC, B-LCL, or PBMC at the numbers indicated in flat-bottomed plates (Flow) for 6 d. In some experiments, DAP.3 B7 transfectants pretreated with mitomycin C were added to the cultures. Wells were pulsed with 1 μ Ci 3 H-TdR before the end of the culture. Proliferation was measured and expressed as described above.

Three Stage Cultures for Tolerance Induction. Purified CD4⁺ T cells (10⁶/well) were plated out in 24-well plates in the presence of allogeneic IFN- γ -treated EC (2 \times 10⁵/well) with or without DAP.3-B7 cells (1:1 ratio), a second population of allogeneic IFN- γ -treated EC expressing a different HLA-DR allele (2 \times 10⁵/well), and B-LCL expressing the same HLA-DR type as the first EC population (2 \times 10⁵/well). After 5 d, the T cells were harvested, purified by isolation on a Ficoll-Paque gradient, and washed five times by low speed centrifugation (210 g for 5 min). Recovered T cells from each culture were subsequently restimulated (2 \times 10⁴/well) with each of the allogeneic IFN- γ -treated EC (2 \times 10⁴/well) in the presence or absence of DAP.3-B7 cells (1:1 ratio), the same B-LCL (2 \times 10⁴/well), and a B-LCL expressing the same HLA-DR allele as the second population of EC (2 \times 10⁴/well). In the rechallenge cultures containing B-LCL, monoclonal anti-DP and anti-DQ antibodies were added at a final concentration of 2 μ g/ml. After 3, 7, and 10 d, cells were pulsed with 1 μ Ci 3 H-TdR. Proliferation was measured and expressed as described above.

Results

Characterization of HUVECs. EC were obtained from four umbilical cord veins. Blood derived from the same cords was used for HLA typing. Once isolated, the EC were depleted of contaminating DR⁺ cells by magnetic bead immunodepletion. No DR⁺ cells were detected by flow cytometric analysis of passaged EC (Fig. 1 a).

To confirm their endothelial lineage, the cells were first stained using the anti-von Willebrand Factor (vWF) mAb, and were tested for acetylated LDL uptake. The cells were all positive for vWF and were able to take up acetylated LDL (data not shown). Before use in functional assays, HUVEC were treated with 1,000 U/ml IFN- γ for 72 h. The expression level of MHC class II molecules induced on the HUVEC was comparable to that of PBMC and B-LCL (Fig. 1, b-d).

To test for expression of the B7 family of costimulatory molecules, EC were stained with the mAbs anti-B7/BB1, which binds to B7.1; BB1, which recognizes B7.1 and has been reported to bind a third isoform of the B7 family of molecule (23); and the fusion molecule CTLA-4-Ig, which binds to all known isoforms of B7. As shown in Fig. 2, no expression of any of these molecules could be detected on

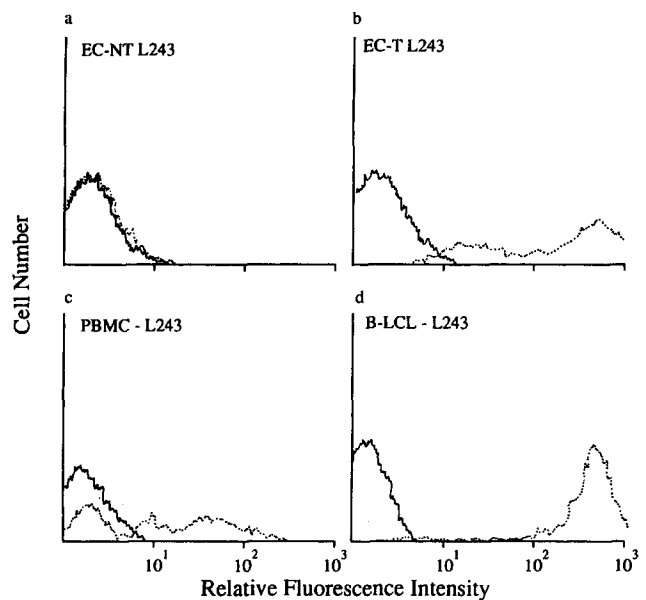


Figure 1. Expression of MHC class II molecules by IFN- γ -treated EC. EC were cultured in the absence (a) or in the presence (b) of IFN- γ (10⁴ U/ml) for 4 d. EC were then stained either with an irrelevant mAb (solid lines) or with anti-DR mAb (dotted lines). The HLA-DR expression of PBMC (c) and a B-LCL (d) are shown for comparison.

HUVEC: in contrast, the B-LCL stained positively with all three anti-B7 reagents. The staining with CTLA-4-Ig is shown in Fig. 1 d. Furthermore, B7 expression was not induced on the EC, neither by cytokine treatment (IFN- γ , TNF- α , and IL-4) nor by preincubation with PMA and/or ionomycin (data not shown).

Antigen Presentation by IFN- γ -treated EC Induced Proliferation by B7-independent T Cell Clones. The antigen-presenting function of HUVEC was first analyzed by testing their capacity to stimulate the proliferation of a panel of five established T cell clones, restricted by DR7, and specific for a defined peptide (307-319) of influenza HA. T cells were purified, as described in Materials and Methods, and their proliferation to IFN- γ -treated EC, or to B-LCL, prepulsed with different doses of peptide, was measured. All the T cell clones proliferated to the peptide presented by EC, although the magnitude of the response was substantially lower than that induced by the B-LCL. An example is shown in Fig. 3 a. The proliferative response could be inhibited by the addition of anti-DR (L243) or anti-LFA-3 (TS 2.9) mAbs to the cultures, but was unaffected by the presence of CTLA-4-Ig, suggesting that the interaction between B7 and CD28/CTLA-4 was not necessary to induce antigen-specific activation of these clones. Confirmation of the B7 independence of these T cell clones was provided by showing that CTLA-4-Ig failed to inhibit proliferation induced by B-LCL prepulsed with optimal or suboptimal doses of peptide (Fig. 3 b).

The Capacity of MHC Class II-expressing EC to Stimulate Primary Alloproliferation by CD4⁺ T Cells Is Inversely Correlated with the Purity of the Responder T Cells. It has been re-

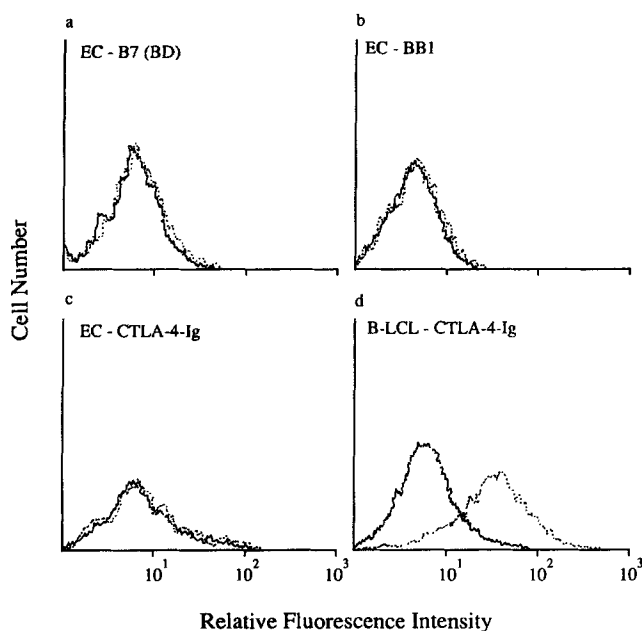


Figure 2. HUVECs lack B7 expression. The results of staining of HUVEC with three anti-B7 reagents, anti-B7.1, BB1, and CTLA-4-Ig (dotted line) and with an irrelevant mAb (solid line) are shown, respectively, in *a*, *b*, and *c*. In *d*, the staining of a B-LCL with CTLA-4-Ig is shown for comparison.

ported that B7-mediated costimulation is critical in the induction of a primary alloresponse by CD4⁺ T cells (8, 24, 25). In this context, the ability of allogeneic IFN- γ -treated EC to induce primary alloproliferation by highly purified resting CD4⁺ T cells from several human leukocyte antigen (HLA)-mismatched donors was examined. CD4⁺ T cells were unable to proliferate to allogeneic EC, while they were strongly responsive to B-LCL and to PBMC expressing the same HLA-DR type as the EC. The results from one of these experiments are shown in Fig. 4 *a*. To test the possibility that the failure of EC to stimulate a primary alloresponse was caused by the lack of B7 expression, CD4⁺ T cells were cocultured with EC in the presence of a murine fibroblast cell line transfected with a cDNA clone encoding human B7.1 (DAP.3-B7). As shown in Fig. 4 *b*, the addition of DAP.3-B7 reconstituted the response of CD4⁺ T cells to EC, by providing *trans* costimulation. The reconstitution was completely abrogated by the addition of CTLA-4-Ig. The purity of the responder T cell population appeared to be inversely correlated with the primary alloproliferative response to EC. This is illustrated by the data shown in Fig. 5. In these experiments, increasing numbers of purified CD4⁺ T cells (up to 3×10^5 /well, the highest number of responder cells used in previous studies [15]) were cultured with 2×10^4 stimulator cells (IFN- γ -treated or untreated EC, as well as B-LCL). In the first experiment (Fig. 5 *a*), a significant response to MHC class II-expressing EC was observed at the highest responder cell numbers. Frozen T cells, stored from the same cell preparation, were then thawed, repurified, and used in an identical prolifera-

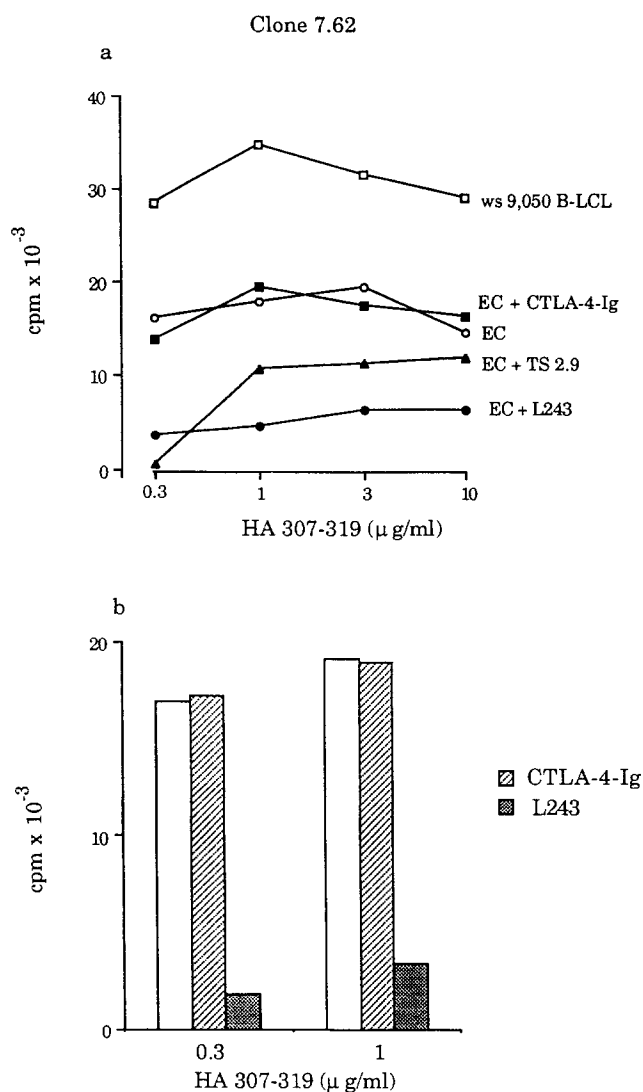


Figure 3. EC can stimulate antigen-specific T cell clones. IFN- γ -treated EC (open circles) and B-LCL (open squares) were pulsed overnight with different doses of peptides. 10^4 /well HA 307-319-specific and DR7-restricted T cell clones were cultured with either 2×10^4 /well prepulsed EC or B-LCL (upper panel). The plates were incubated for 3 d and ^3H -TdR was added for the last 18 h. Results are expressed as the mean cpm for triplicate cultures $\times 10^{-3}$. T cell proliferation was inhibited by the addition of anti-DR (solid circles) or anti-LFA3 (solid triangles), but not by CTLA-4-Ig (solid squares). Proliferation by the T cell clone to B-LCL prepulsed with suboptimal (0.3 $\mu\text{g/ml}$) or optimal (1 $\mu\text{g/ml}$) doses of peptide was inhibited by the addition of anti-DR mAb (1 $\mu\text{g/ml}$), but not by CTLA-4-Ig (1 $\mu\text{g/ml}$).

tion assay (Fig. 5 *b*). After this second round of T cell purification, the previously observed proliferative response to IFN- γ -treated EC disappeared, even at high responder cell numbers, while the response to the B-LCL was slightly increased.

A summary of 10 experiments using 8 different responder/stimulator combinations is shown in Table 1, in which the responses of purified CD4⁺ T cells to allogeneic IFN- γ -treated EC, untreated EC, PBMC, and B-LCL are compared. The proliferation to PHA (2 $\mu\text{g/ml}$) by PBMC and

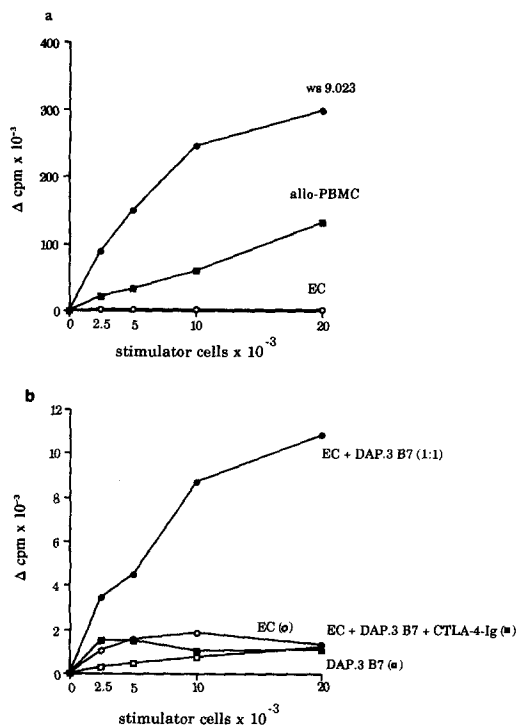


Figure 4. EC are unable to stimulate a primary MLR. CD4⁺ T cells from a DR15/DR7 heterozygous individual were purified as described in Materials and Methods. CD4⁺ T cells (10^5 cells/well) were cultured with different numbers of HLA-DRB1*1701 expressing B-LCL (solid circles), PBMC (solid squares), and EC pretreated with IFN- γ (10^4 U/ml) (open circles). The results are shown in the upper panel. In the lower panel, CD4⁺ T cells were cultured either with EC alone (open circles) or with the DAP.3 cell line expressing B7 (open squares). In addition, CD4⁺ T cells were cocultured with EC and DAP.3-B7 cells in the absence (solid circles) or in the presence (solid squares) of CTLA-4-Ig. The plates were incubated for 5 d and ³H-TdR was added for the last 18 h. Results are expressed as the mean cpm for triplicate cultures $\times 10^{-3}$, corrected for background proliferation of both T cells and stimulators alone (Δ cpm). SEs were routinely <10%.

purified CD4⁺ T cells is also shown. Notably, in the two experiments in which significant proliferative responses to allogeneic IFN- γ -treated EC could be detected, the PHA responses of the purified CD4⁺ T cells were also high. This was indicative of the presence of contaminating autologous APCs in the T cell populations in these two experiments.

It has been suggested that the CD45RO⁺ subset of CD4⁺ T cells is more efficiently stimulated by EC than the CD45RA⁺ subset (25). The proliferation of separated CD45RO⁺ and CD45RA⁺ T cell subpopulations to HLA-DR-expressing allogeneic EC was compared. Neither subset proliferated to the EC, while a strong response to B-LCL was observed (Fig. 6 a). Taken together, these data suggest that the ability of EC to stimulate a primary MLR is correlated with the presence of contaminating autologous APC in the responder T cell population.

CD4⁺ CD45RO⁺ Resting T Cells were Neither "Primed" Nor "Anergized" by Coculture with IFN- γ -treated Allogeneic EC. It has previously been shown that specific recogni-

tion in the absence of B7-mediated costimulation can lead to a state of nonresponsiveness in T cell clones. Work by Tan and colleagues (27) showed that in a unidirectional primary MLR, responding T cells can be rendered refractory to a subsequent challenge with the same stimulator cells by the presence of CTLA-4-Ig in the primary cultures. In a primary MLR model by Boussiotis and colleagues (25), murine cells transfected with human HLA-DR molecules induced alloantigen-specific anergy in purified CD4⁺ T cells, and cotransfection of human B7 in the same stimulator cells resulted in their ability to costimulate proliferation of the alloreactive T cells. It has also been shown that both memory and naive T cells require two signals to be activated, although naive T cells have a higher threshold for both signals (28). We reasoned that this quantitative difference might be reflected in a different susceptibility to the induction of unresponsiveness. This possibility was examined in a three-step culture model. Purified CD4⁺ T cell subsets were cocultured with two populations of allogeneic IFN- γ -treated EC and a B-LCL expressing the same HLA-DR alleles as the first population of EC. The results of a simple proliferation assay, performed in parallel with the primary cocultures, is shown in Fig. 6 a. After 5 d, the T cells were harvested and purified as described in Materials and Methods. T cells from each culture were subsequently rechallenged in a proliferation assay with both populations of allogeneic IFN- γ -treated EC in the presence or absence of DAP.3-B7 cells, the same B-LCL, and a second B-LCL expressing the same HLA-DR allele as the second population of EC. A representative example of a series of criss-cross experiments is shown in Fig. 6 b. CD45RO⁺ T cells that had been cocultured with IFN- γ -treated EC were still able to respond to EC plus DAP.3-B7 or B-LCL (expressing the same DR as the EC), but were not "primed" in that the proliferative response had a primary kinetic (peak of response after 7–10 d; Fig. 6 b, i and ii). T cells previously cocultured with B-LCL were "primed," and responded with a secondary kinetic to EC and to the same B-LCL used for priming (peak of response after 3 d; Fig. 6 b, iii).

Coculture of CD4⁺ CD45RA⁺ T Cells with IFN- γ -treated Allogeneic EC-induced Allospecific Nonresponsiveness. In contrast with the results obtained with CD4⁺ CD45RO⁺ T cells, after being cultured in the presence of MHC class II-expressing EC, CD45RA⁺ T cells lost the ability to respond to the same allogeneic EC in the presence of the B7-transfected DAP.3 cells. The response to B-LCL expressing the same DR type as the EC was also severely depressed, while the T cells retained the capability of responding to other IFN- γ -treated EC in the presence of B7-transfected cells and to another B-LCL (Fig. 6 c, i and ii). As previously shown for the CD45RO⁺ subset, the CD45RA⁺ T cells were primed after their culture with B-LCL and proliferated with a peak of response at day 3 to both EC and B-LCL expressing the same HLA-DR molecule as the B-LCL used for the priming (Fig. 6 c, iii). Anti-DP and -DQ mAbs were included in the rechallenge cultures containing B-LCL to exclude possible effects of DP and DQ differences between the EC and the B-LCL.

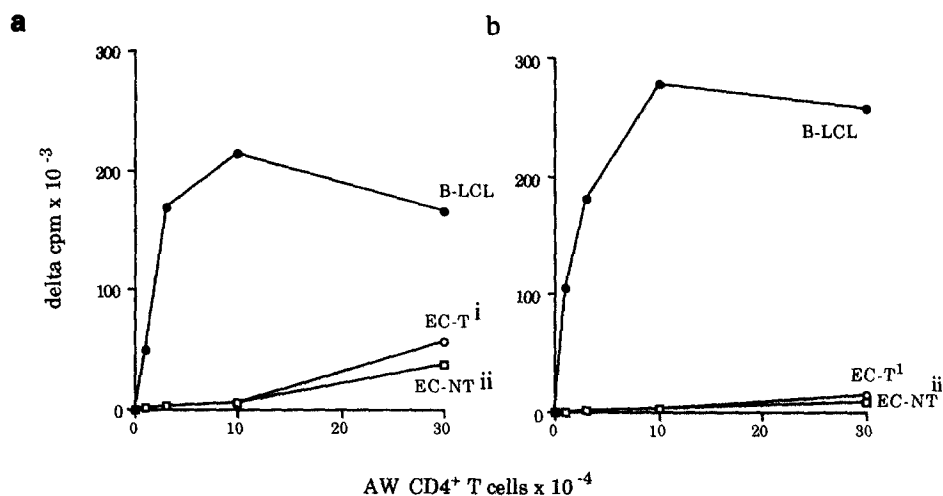


Figure 5. The primary alloproliferative response to MHC class II-expressing EC is inversely correlated with the purity of the responder CD4⁺ T cell population. CD4⁺ T cells from a DR4/15 heterozygous individual (AW) were purified as described in Materials and Methods. Increasing numbers of CD4⁺ T cells were cultured with 2×10^4 HLA-DRB1*1701 expressing B-LCL (solid circles), EC pretreated with IFN- γ (10^4 U/ml) (open circles), and untreated EC (open squares). In a, the results of an assay after a poor CD4⁺ T cell enrichment (response of 10^5 T cells to $2 \mu\text{g/ml}$ PHA = 35.1×10^3 cpm) are shown. Frozen T cells from the same preparation were then thawed, repurified after the same procedure and used in an identical

primary MLR assay. The results are shown in b (response to PHA after the second purification = 22.0×10^3 cpm). The plates were incubated for 5 d, and $^3\text{H-TdR}$ was added for the last 18 h. Results are expressed as the mean cpm for triplicate cultures $\times 10^{-3}$, corrected for background proliferation of both T cells and stimulators alone (Δ cpm). SEs were routinely $<10\%$. *, IFN- γ -treated EC; *NT, non-IFN- γ -treated EC.

In the Presence of B7 trans Costimulation, Coculture of CD45RO⁺ and CD45RA⁺ T Cells with IFN- γ -treated Allogeneic EC Led to Allospecific Priming. In previously described culture models, in which T cell nonresponsiveness was induced by cognate interactions with costimulation-deficient stimulators, the T cells could be protected from the induction of nonresponsiveness by providing B7-mediated co-

stimulation (8). This possibility was tested by coculturing CD45RO⁺ and CD45RA⁺ T cell subsets with IFN- γ -treated EC in the presence of B7-transfected cells. As is shown in Fig. 7, a and b, both T cell subsets were primed by EC in the presence of B7-mediated costimulation supplied in the form of B7 transfectants, and they responded to a rechallenge with the same EC plus DAP.B7 with a sec-

Table 1. IFN- γ -treated EC Fail to Induce Primary Alloresponses by CD4⁺ T Cells

Exp.	Comb.*	Stimulator cells used				Response to PHA (2 $\mu\text{g/ml}$)	
		EC-T [‡]	EC-NT [‡]	PBMC [§]	B-LCL [§]	T CD4 ⁺	PBMC
17.2.94	FM/EC F5	0.7	ND	21.8	63.9	25.0	125.9
17.2.94	RG/EC F5	0.6	ND	7.9	52.5	20.3	99.8
31.3.94	RG/EC F4	1.1	ND	26.0	89.0	15.2	89.0
11.7.94	AW/EC F4	5.9	6.2	43.1	214.7	35.1	105.6
17.7.94	AW/EC F4	2.6	2.9	50.7	186.4	22.0	112.4
14.7.94	RH/EC F3	1.5	1.4	94.0	252.2	12.0	ND
20.7.94	RH/EC F4	1.3	1.4	132.9	301.4	10.0	98.1
12.8.94	FM/EC F2	15.5	1.7	93.2	275.9	37.9	102.3
12.9.94	RH/EC F4	0.2	0.2	ND	189.1	19.4	103.5
23.11.94	RL/EC F3	3.3	5.3	47.8	110.0	16.8	89.4

Summary of 10 experiments using 8 different combinations showing the proliferative responses of purified CD4⁺ T cells (10^5 /well to 2×10^4 allogeneic IFN- γ -treated EC (column 3), untreated EC (column 4), PBMC (column 5), and B-LCL (column 6). In the last two columns, the proliferative responses of 10^5 purified CD4⁺ T cells and PBMC to PHA (2 $\mu\text{g/ml}$) are shown. Results are expressed as the mean cpm for triplicate culture $\times 10^{-3}$, corrected for background proliferation of both T cells and stimulators alone (Δ cpm). SEs were routinely $<10\%$.

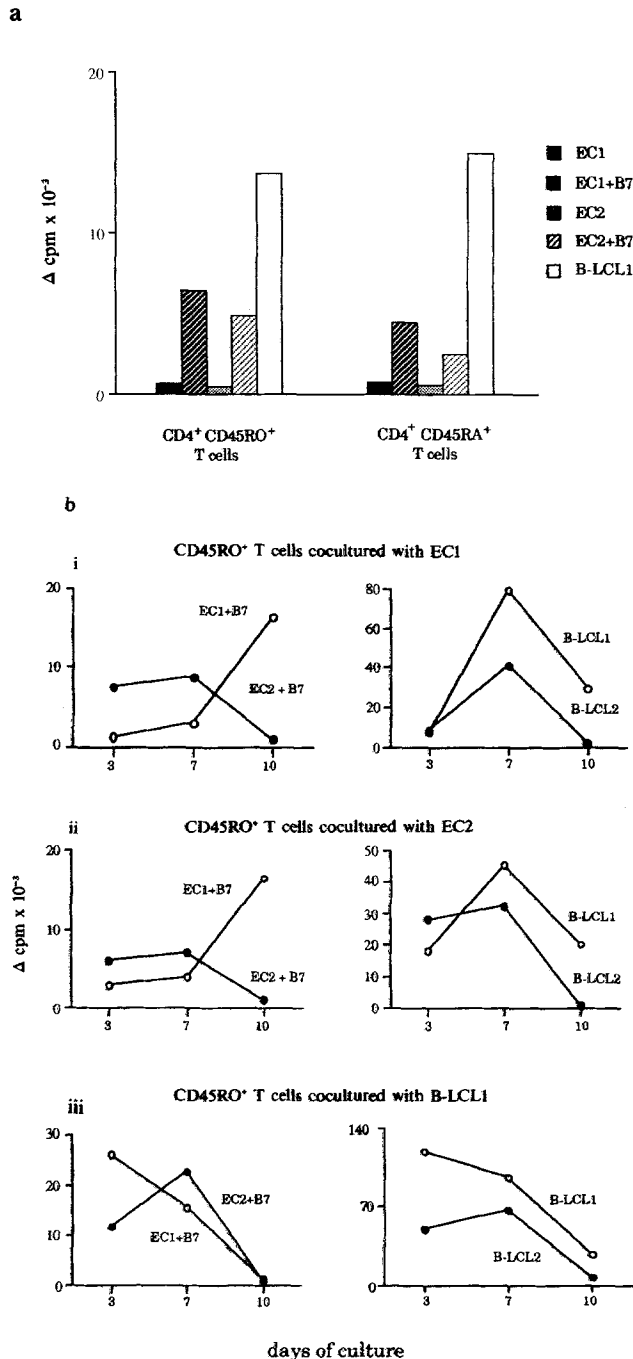
*Responder/stimulator combination.

[‡]EC treated (T) or not treated (NT) with IFN- γ .

[§]Allogeneic PBMC and B-LCL expressing the same DR alleles as the EC.

^{||}Proliferative responses to PHA by 10^5 CD4⁺ T cells after purification and 10^5 PBMC before purification.

ondary kinetic. Furthermore, CD45RA⁺ T cells cocultured with IFN- γ -treated EC in the presence of anti-DR mAb remained capable of responding to a subsequent stimulation with the same EC and B7-transfected cells without being primed (Fig. 7 c). This demonstrates that the induction of the allospecific nonresponsiveness was strictly dependent on the engagement of the T cell receptor by the allogeneic HLA-DR molecules on the EC in the absence of costimulation.



Discussion

In this study, the antigen-presenting cell function of HUVEC induced to express MHC class II molecules by IFN- γ treatment was investigated. The results obtained showed that EC can be competent APC for human T cell clones, but are unable to induce primary alloreactive re-

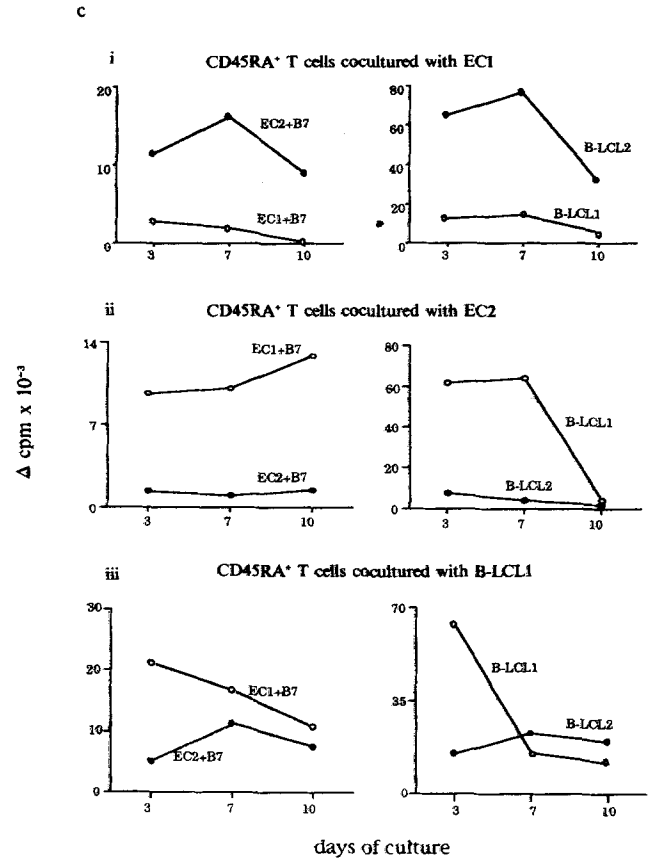


Figure 6. Antigen recognition on EC by CD45RA⁺ T cells renders them unresponsive to a subsequent rechallenge. (a) Alloproliferation of freshly purified CD45RO⁺ and CD45RA⁺ CD4⁺ T cells to HUVEC and B-LCL during the first step of the tolerance induction experiments. T cells (10^4 /well) from either of the two subsets responded to allogeneic B-LCL (10^4 /well), but not to HUVEC (10^4 /well), unless *trans* costimulation was provided by the addition of DAP.3-B7 cells (1:1 ratio). (b and c) Purified CD4⁺ T cell subsets, CD45RO⁺ (b) and CD45RA⁺ (c), were cocultured with allogeneic IFN- γ -treated EC 1 (DR17), EC 2 (DR7/DR13), and B-LCL 1 (expressing the same HLA DR as the EC 1), as described in Materials and Methods. T cells from each culture were subsequently rechallenged in a proliferation assay with EC 1 (10^4 /well) and EC 2 (10^4 /well) in the presence of DAP.3-B7 (1:1 ratio) cells, B-LCL 1 (10^4 /well), and B-LCL 2 (DR7) (10^4 /well). In the rechallenge cultures containing B-LCL, the B7.21 (anti-DP) and L2 (anti-DQ) mAbs were added to exclude any effect of mismatching between the EC and the B-LCL at these loci. Rechallenge cultures were harvested on days 3, 7, and 10 to detect responses with primary versus secondary kinetics.³H-TdR was added for the last 18 h. Results are expressed as the mean cpm for triplicate cultures $\times 10^{-3}$, corrected for background proliferation of both T cells and stimulators alone (Δ cpm). SEs were routinely $<10\%$. The responder and stimulator cells present in the initial culture are indicated above each pair of graphs. The stimulator cells used in the rechallenge step are indicated within each graph.

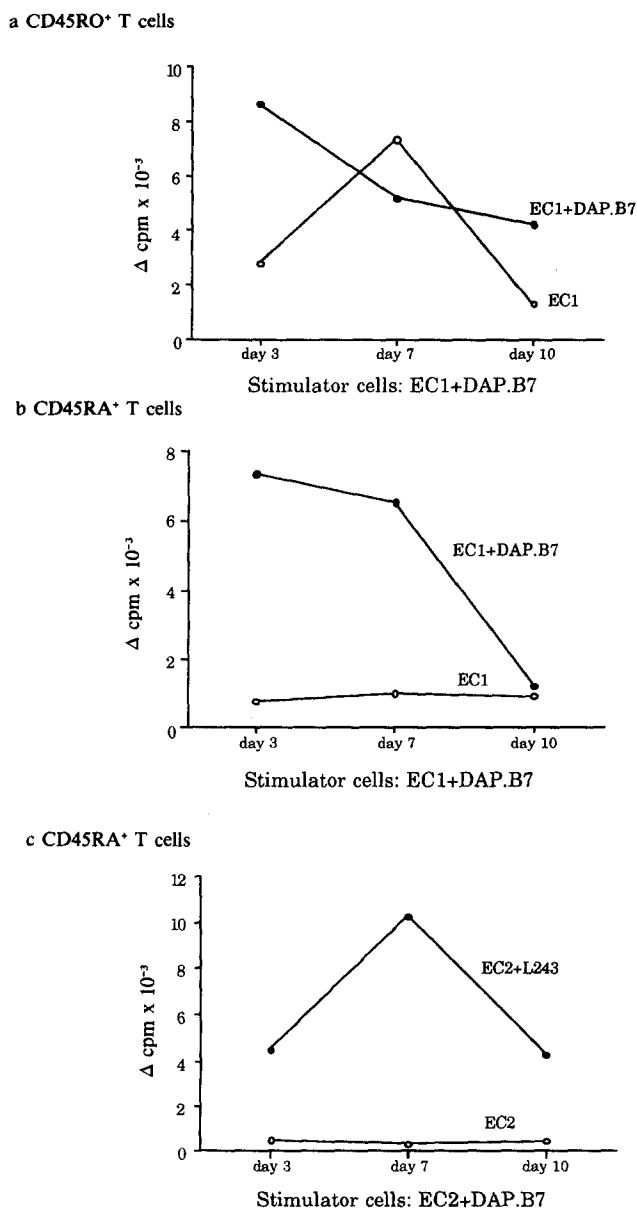


Figure 7. Delivery of B7-mediated costimulation results in “priming” of both T cell subsets and blockade of cognate interaction rescues CD45RA⁺ T cells from the induction of allospecific nonresponsiveness. Purified CD4⁺ T cell subsets, CD45RO⁺ (a) and CD45RA⁺ (b), were cocultured with allogeneic IFN- γ -treated EC 1 (DR17) in the absence or presence of B7-transfected cells, as described in Materials and Methods. (c) CD45RA⁺ T cells were cultured with EC 2 in the absence or presence of L243 (1 μ g/ml). T cells from each culture (10^4 /well) were subsequently rechallenged in a proliferation assay with the same EC (10^4 /well) in the presence of DAP.3-B7 cells (1:1 ratio). Rechallenge cultures were harvested on days 3, 7, and 10, and 3 H-TdR was added for the last 18 h. Results are expressed as the mean cpm for triplicate cultures $\times 10^{-3}$, corrected for background proliferation of both T cells and stimulators alone (Δ cpm). SEs were routinely $<10\%$. The stimulator cells present in the initial culture are indicated within each graph. The stimulator cells used in the rechallenge step are indicated under each graph.

responses. The inability of EC to induce primary T cell responses was overcome by the addition of B7.1-expressing transfectants, suggesting that the EC were unable to provide costimulation. Furthermore, recognition of alloantigen on the surface of EC induced nonresponsiveness in resting CD45RA⁺ T cells.

The HUVEC were phenotyped using a variety of mAbs specific for EC-specific, accessory, and MHC class II molecules. After IFN- γ induction, levels of MHC class II molecule expression comparable to that of B-LCL were achieved. In addition, the EC expressed substantial levels of LFA-3 and intercellular adhesion molecule-1. Most significantly, however, no expression of the B7 family of molecules was detected using several anti-B7 reagents. This finding is consistent with the contention that B7 expression is confined to specialized, bone marrow-derived APC.

The capacity of the EC to induce proliferation by the T cell clones used in these experiments correlates with the apparent B7 independence of the T cells. In our experience, $\sim 70\%$ human T cell clones do not require B7-mediated costimulation to secrete IL-2 (Hargreaves, R., et al., manuscript in preparation). Whether B7 dependence correlates with TCR avidity, use of an alternative costimulatory pathway or some other feature of the T cell is unclear at the present time. The key issue is the extent to which secondary T cell responses are B7 dependent in vivo. If they are not, this would raise the possibility that previously activated T cells may be further activated during the process of transmigration across the EC lining small blood vessels. This could apply in the context of local inflammation when the EC have been induced to express MHC class II molecules, and may be displaying antigenic peptides at the cell surface.

The failure of EC to induce primary T cell responses, as observed here, contrasts with previously published results from several groups. There are at least two possible explanations for these different results. First, the EC used in the experiments described here were depleted of any constitutively MHC class II⁺ cells by magnetic bead depletion, before induction of MHC class II expression with IFN- γ . It is conceivable that primary cultures of EC may contain small numbers of bone marrow-derived cells, such as dendritic cells (29). Given the potency of these cells in inducing primary T cell responses, numbers below the limits of detection by flow cytometry can have major functional effects. Second, differences may exist in the purity of the responder T cell populations. The potential relevance of T cell impurity is illustrated by the finding reported here, that responsiveness to allogeneic EC correlated with responsiveness of the T cells alone to PHA. Given that mitogen responses are dependent on the presence of accessory cells, these results suggest that reactivity of naive T cells to allogeneic EC may depend on low level contamination of the responder T cells with accessory cells.

In previous studies, however, although great care was taken to eliminate MHC class II-bearing cells from the responder T cell populations, the opposite result was obtained (15, 16). The reasons for this discrepancy remain unclear.

The consequence of alloantigen recognition on EC was further investigated by measuring the responses of peripheral blood T cell subpopulations expressing the two major isoforms of CD45, CD45RA, and CD45RO. Neither population showed any significant proliferation to the MHC class II-expressing EC. This led to examination of the consequences of alloantigen recognition on EC for the two subpopulations of peripheral blood T cells. For the RO⁺ population, this appeared to be a neutral encounter, in that rechallenge with the same EC in the presence of *trans* costimulation led to a proliferative response with the kinetics of a primary response. This is an unusual occurrence; under most circumstances, specific recognition by T cells either induces activation of an effector function, or leads to some form of T cell silencing. For the RA⁺ population, in contrast, recognition of alloantigen presented by the EC induced a marked state of reduced reactivity to a second challenge with the same MHC alloantigens expressed either by the same EC or by a B cell line.

In physiological terms, these findings make reasonable sense. Resting RA⁺ T cells have low level expression of accessory molecules, and T cells with a given specificity are present in the naive T cell repertoire at a very low fre-

quency. As a consequence, it is unlikely that interactions between MHC class II⁺ EC and RA⁺ CD4⁺ T cells will occur. For previously activated T cells, either in a resting or in a recently activated state, interactions with activated EC may well occur. The outcome of such interactions may either be neutral or may lead to further activation of the T cell as it enters the inflamed tissue.

Underlying all these interpretations are the unresolved questions as to why EC express MHC class II molecules at all, and what are the peptides that they display. One possibility is that the EC at the site of inflammation, once induced to express MHC class II molecules, process and present peptides derived from the pathogens causing inflammation in the underlying tissue. This would create the possibility of further activating antigen-specific T cells as they enter into the inflamed tissue. It is also conceivable that the activated EC acts as a "shop window," displaying, in MHC molecule-bound peptide form, a sample of the antigens within the underlying tissue. Although T cell recruitment is largely antigen nonspecific, cognate recognition by trafficking T cells may allow a degree of specific recruitment.

Testing the physiological significance of the observations made here will require the use of *in vivo* models.

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