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ORIGINAL ARTICLE

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The active role played by xenogeneic endothelial cells in the indirect presentation pathway is not lymphocyte *trans*-co-stimulation

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Abstract The human $CD4^+$ T lymphocyte response to major histocompatibility complex (MHC) class II-negative porcine endothelial cells is dependent on the presence of human monocytes through a human leukocyte antigen (HLA) class II-restricted indirect presentation pathway. Because the role of porcine endothelial cells had been previously shown to do more than simply supply xenopeptides, co-stimulatory signals were analysed. Endothelial cells were shown to express the CD54, CD58, CD59 and CD86 transcripts; however, no membrane B7 molecule could be detected. Blocking experiments in a direct pathway model confirmed that porcine endothelial cells could provide co-stimulatory signals to human T cells through the CD2 and LFA-1 pathways. Nevertheless, the proliferation achieved in the indirect presentation model required co-stimulation by LFA-1, CD2 and CD28, engaged by co-stimulation molecules expressed in the *cis*-form by the human monocytes. These results clearly show that the active role played by the endothelial cells in the indirect pathway is not lymphocyte *trans*-co-stimulation and suggest that*cis*-co-stimulation dominates *trans*-co-stimulation when both are present.

Keywords Endothelial cells
cis-co-stimulation · Xenotransplantation

Introduction

The indirect presentation pathway, i.e. the presentation of graft-derived peptides by recipient antigen-presenting cells (APCs) has, in recent years, been found to play a major role in chronic allograft rejection [1, 2, 3]. One of the deleterious consequences of the indirect responses is the ability of elicited T cells to co-operate with anti-graft B cells, inducing T cell-dependent anti-graft humoral responses [4]. The occurrence of chronic rejection, despite immunosuppression, witnesses that the indirect presentation pathway is hard to control therapeutically. The consequences of the indirect presentation pathway would probably be even worse in xenotransplantation, due to the huge number of divergent peptide motifs that could be presented by host APCs and recognised by recipient T cells [5].

The transplantation of transgenic pig organs into primates always leads to delayed vascular [6] or cellular [7] rejections in the few weeks following grafting, despite adsorption of xenoreactive natural antibodies [8, 9] and a strong T cell- and B cell-targeted immunosuppression [10]. These therapeutic procedures frequently fail to prevent the reappearance of anti-pig antibodies, frequently of anti-αGal specificity [9], and the occurrence of an associated vasculopathy. These anti-αGal antibody responses are dependent on strong help by xenogeneic T cells specific for xenopeptides captured, processed and presented by αGal-specific B cells, through an indirect presentation pathway [5, 11].

The indirect pathway is more easily shown in xenogeneic rather than in allogeneic in vitro models, probably because of a higher precursor frequency. In particular, by the use of porcine aortic endothelial cells (PAECs) devoid of any swine leucocyte antigen (SLA) class II molecules, the induced human $CD4^+$ T lymphocyte ($huCD4^+$) proliferation was previously demonstrated to be strictly dependent on the presence of monocytes and to be human leucocyte antigen (HLA) class II-restricted [12] (Fig. 1). Moreover, simply supplying endothelial cell extracts or xenogeneic material in the form of other adherent porcine cells did not lead to T cell proliferation in the presence of human monocytes, indicating an active role of PAECs in this process [12].

Because PAECs express co-stimulatory molecules able to act across the species barrier [13, 14, 15], our hypothesis was that PAECs could contribute to indirect T cell responses by providing co-stimulatory signals to T cells (*trans*-co-stimulation) (Fig. 1).*Trans*-co-stimulation is defined as co-stimulation provided by a bystander cell when antigen presentation occurs in a context of low co-stimulatory molecules on the APCs, namely lack of B7 co-stimulatory molecules [16, 17, 18, 19]. To determine if co-stimulation is provided in the *trans*-configuration in this xenogeneic mixed lymphocyte endothelial cell culture (XMLEC) model, the panel of co-stimulatory molecules expressed by porcine endothelial cells was first analysed at the transcript level by reverse transcriptase–polymerase chain reaction (RT–PCR) experiments based on primers derived from established or newly determined cDNA sequences.

Co-stimulation-blocking experiments were, thereafter, performed in PAEC/$huCD4^+$ co-cultures in the presence of phytohemagglutinin (PHA), a simplified model mimicking the direct presentation and conducive to the study of endothelial co-stimulation [20]. Once we had determined the endothelial co-stimulatory pathways functionally active in this simplified model, we performed blocking experiments in the indirect pathway-based model (XMLEC) [12] with species-specific reagents, which allowed us to distinguish the porcine (*trans*-) or human (*cis*-) origin of the co-stimulation. Thus, we demonstrate that all the co-stimulatory signals operating in the classical model originate in*cis*-form from the human adherent APCs (huAPCs), refuting the *trans*-co-stimulation hypothesis, despite the presence of cross-reactive co-stimulatory molecules on PAECs.

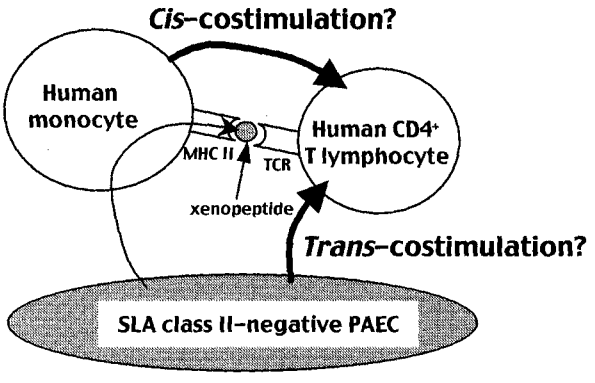

Fig. 1 Indirect presentation pathway and hypothesis concerning the co-stimulatory signals

Materials and methods

Porcine cells

PAECs were isolated from miniature swine homozygous for the swine leucocyte antigen (d) haplotype, as has been previously described [21]. Briefly, PAECs were harvested after treatment of aortas with collagenase A from *Clostridium histolyticum* (BoehringerMannheim, Meylan, France). They were seeded in 25 cm^2gelatin-coated culture flasks in RPMI-1640 medium (Life Technologies, Cergy-Pontoise, France) supplemented with 10% heat-inactivated foetal calf serum (FCS) (Life Technologies), 25 mM sodium bicarbonate (Flow, LesUlis, France), 2 mM L-glutamine (Flow), 1 mM sodium pyruvate (Flow), 60 µg/ml tylocin (Life Technologies), 50 IU/ml penicillin (Flow) and 50 µg/ml streptomycin (Flow). PAECs were subcultured after trypsin–EDTA (Life Technologies) treatment and used from the second to the eight subcultures.

Human cells

Human peripheral blood mononuclear cells (PBMCs) from healthy volunteers were isolated by centrifugation (20 min, 800 *g*) of heparinized blood over a d = 1.077 lymphocyte separation medium (Lymphoprep; Nycomed, Oslo, Norway), and cells were collected from the plasma/Lymphoprep interface. To prepare highly purified $CD4^+$ lymphocytes, we mixed huPBMCs with anti-CD4-coated magnetic beads (Dynabeads; Dynal, Oslo, Norway) at a bead:cell ratio of 3:1. After incubation at 4°C with gentle end-over-end mixing for 45 min, we washed the rosetted cells five times in phosphate buffered saline (PBS) supplemented with 2% FCS, using a magnet to retain the rosettes, and then re-suspended them in 100 µl RPMI-1640 containing 10 µl DetachA-Bead (Dynal) for 30 min at room temperature to free the lymphocytes from the magnetic beads.

HuAPCs, taken from the same individual as the $CD4^+$ T cells, were isolated by a procedure described by Freundlich and Avdalovic [22]. Briefly, huPBMCs were incubated for 45 min at 37°C in a plastic culture flask pre-coated with bovine gelatin (Sigma, St Quentin-Fallavier, France) and autologous plasma. After washing

out non-adherent cells we collected the huAPCs, following their incubation with a cold solution of 5 mM EDTA in RPMI-1640–10% FCS for 30 min at 4°C. This cell population always contained more than 80% $CD14^+$ cells, as determined by flow cytometry analysis.

For the human dendritic cell (DC) generation, PBMCs were seeded into a 175 cm^2 culture flask (Becton Dickinson, Le Pont-de-Claix, France) in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 2 mmol glutamine, 50 IU/ml penicillin and 50 μg/ml streptomycin. After incubation at 37°C for 45 min, non-adherent cells were removed, and the adherent cells were cultured for 7 days with 1,000 IU/ml GM-CSF (Leucomax; Schering-Ploug, LevalloisPerret, France) and 250 IU/ml IL-4 (R&D Systems) to generate immature DCs. Mature DCs were obtained after two additional days of culture in the presence of 800 IU/ml TNF-α (R&D Systems).

Xenogeneic mixed cultures

XMLEC was performed as previously described [23]. Briefly, we seeded the PAECs in triplicate in 96-well flat-bottomed culture plates (Falcon 3072; Becton Dickinson) to obtain confluent monolayers (3×10^4 cells/well). In the simplified "two-cell" model, $huCD4^+$ were cultured for 3 days in the presence of PHA (0.05 μg/ml; Sigma), either alone or on porcine adherent cell monolayers. In the XMLEC model, 10^5 huAPCs were first added to PAECs and both were irradiated (30 Gy). Then, 10^5 $huCD4^+$ were added to the wells for a 6-day co-culture, at 37°C in a humidified 5% CO_2 incubator. One microcurie (3.4×10^4 Bq) of [^{3}H]-thymidine (Amersham, Little Chalfont, UK) was added to each well 18 h before the end of incubation. Tritiated thymidine incorporation and cell proliferation were quantified with an automated harvester (Filtermate 196; Packard, Rungis, France). Radioactivity on filter plates was measured with a liquid scintillation beta-counter (Tri-Carb 2550 TR/ll; Packard). Results were expressed in counts per minute as the mean ± SD of thymidine incorporation in triplicate wells.

Antibodies and fusion proteins

The following murine monoclonal antibodies (mAbs) were used in the blocking experiments: T11.2 (mIgG1, anti-huCD2) (Beckman Coulter), AICD58 (mIgG2a, anti-huCD58, Beckman Coulter), BT3 (mIgG2a, anti-huCD28) (Diaclone, Besançon, France), IT2–2 (mIgG2b anti-huCD86, Pharmingen, Becton Dickinson), CD80 (a gift from Prof. D Olive, France), AFOL1 (mIgG1, anti-huCD11a) (IMTIX, Marcy l'Etoile, France) and 84H10 (mIgG1, anti-huCD54, Beckman Coulter). All mAbs were dialysed against RPMI-1640 before use in blocking experiments. Two recombinant human CTLA4-Fc fusion proteins were used. The first was purchased from R&D systems (Minneapolis, Minn., USA) and the second was kindly provided by Dr P. Linsley (Bristol-Myers-Squibb, N.Y., USA). The human IgG Fc fragments used as controls were kindly provided by Dr M.C. Bonnet, (Pasteur Mérieux, Marcy l'Etoile, France). All blocking agents were used at saturating concentrations. Secondary fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG (Fc) antibody (1/1500) (Cappel, Organon, Teknica, Turnhout, Belgium) was used to reveal the binding of the human CTLA4-Fc fusion protein.

Flow cytometry analysis

After purification of $huCD4^+$ T cells and preparation of adherent huAPCs, the expression of CD4 and CD14 were determined with mAbsBL4 (mIgG2a, anti-CD4; Beckman Coulter) and RMO52 (mIgG2a, anti-CD14; Beckman Coulter) directly conjugated to phycoerythrin. We incubated 5×10^5 cells with these mAbs or their isotype-matched control for 30 min at 4°C. After two washes in PBS, the cells were fixed in 0.5% paraformaldehyde until analysis.

In order to verify the absence of B7 membrane expression on the PAECs, we stained the PAECs with two different human CTLA4-Fc fusion proteins, in comparison with L23 cells as positive controls. We incubated 5×10^5 cells with CTLA4-Fc or Fc fragments for 30 min at 4°C. After two washes in PBS, the cells were incubated with an FITC-conjugated goat anti-human IgG1 (Beckman Coulter) for 1 h at 4°C. After two washes in PBS, the cells were immediately analysed by flow cytometry.

All flow cytometry analyses were performed with a 488 nm laser flow cytometer (FACScalibur; Becton Dickinson). Multivariance analysis was performed with CellQuest software (Becton Dickinson).

Reverse transcriptase–polymerase chain reaction

We prepared total mRNA from resting PAECs and from XMLEC co-cultures by using the Dynabeadsmrna Direct kit (Dynal) and following the manufacturer's instructions. Total mRNA from the L23 porcine lymphoblastoid cell line, which expresses large amounts of co-stimulatory molecules [24], was used as a positive control. Total mRNA of human mature monocyte-derived dendritic cells, after sequential treatment by GM-CSF/IL-4 and TNF-α, was used as a negative control. Total mRNAs were then reverse-transcribed in a 50 μl reaction mixture containing

Table 1 Analysis of porcine co-stimulatory molecules by RT–PCR

Molecules	Forward and reverse primers 5′ → 3′	PCR reaction (bp)	GenBank	PCR product size	Reference
CD80	catcgttcaggtgaccaaaacagtg agccaggatcacaatgcagaggtta	55°C 30 s	AF203443	214	[31]
CD86	aaatgtgagcatcgtctgtgtcctg ggtttcaccacattcattagagggg	55°C 30	L76099	235	[32]
CD54	actacagtgagcatagagtg aacactgcccaagatagcca	47°C 1 min	AF156712	515	[33]
CD48	tcacatgctggtcctggatcaca gcttacaggattgctgacttggc	60°C 1 min	X13016 (rat)[a] M59904 (human)[a]	175	A. Brossay et al., unpublished data
CD58	aaaggccggaccgccatggcct cctccctcgtggcctaccagtgaa	60°C 1 min	AF469666	912	GenBankAF469666
CD59	acctagcggagaagaagctgaag ctaggtttagtccttcccaacagg	55°C 30 s	AF020302	258	[34]
GAPDH	acagtccatgccatcactgcc gcctgcttcaccaccttcttg	60°C 30 s	J04038	280	[35]
β2-microglobulin	ttttcacaccgctccagtag gatccacagcgttaggagt	62°C 30 s	L13854	308	[36]

[a]Sequences used to design consensual primers

500 μM of each deoxynucleotide, 4 μM oligo$(dT)_{20}$, 25 U RNAse inhibitor and 25 U AMV reverse transcriptase (BoehringerMannheim, Meylan, France). After 1 h of incubation at 42°C, the enzyme was inactivated at 95°C for 5 min. PCR amplification was performed in a total reaction volume of 25 μl containing 25 mM $MgCl_2$, 0.5 U Taq polymerase and 200 μM of each reverse and forward oligonucleotide specific primer (Table 1) synthesized by Genset (Paris, France).

PCR was set up in a GeneAmpPCR system 2400 (Perkin Elmer France SA, Saint Quentin en Yvelines, France) programmed for an initial denaturation step of 3 min at 94°C, followed by 35 cycles at 94°C for 30 s, hybridisation for 30 s or 1 min at various temperatures (Table 1) and 72°C for 1 min. The final extension step was performed at 72°C for 7 min. PCR products were then analysed by electrophoresis in 1.6% agarose gel run in TBE buffer (90 mM Tris-HCl, 90 mM boric acid, 2.5 mM EDTA) (Euromedex, Mundolsheim, France) containing 1 μg/ml ethidium bromide (Eurobio). Gels were ultraviolet transilluminated (Gel Doc 1000 system, Bio Rad, Ivry sur Seine, France), and the picture was captured by means of Kodak Digital Science Image (Rochester, N.Y., USA).

Results

Expression of co-stimulatory molecule mRNAs in PAECs

In the absence of mAbs to detect the porcine co-stimulatory molecules, RT–PCR experiments were performed with resting PAECs and PAECs from 24 h and 72 h XMLEC co-cultures. Specific oligonucleotide primers were specifically designed to study porcine B7 molecules (CD80 and CD86), porcine CD54, and the three putative porcine ligands of human CD2, i.e. poCD48, poCD58 and poCD59 (Table 1). Because cDNA extracted from XMLEC co-cultures originated from both porcine (PAEC) and human (PBMC) cells, the amount of total cDNA was quantified with primers that amplify both human and porcine GAPDH transcripts, and the amount of porcine cDNA by primers that amplify only porcine β2-microglobulin transcripts (S. Dall'ozzo, P. Reverdiau, S. Iochmann, A. Brossay, Y. Gruel, unpublished work). As expected, the L23 cells used as positive controls did express all seven transcripts, whereas no amplification was detected with human dendritic cells, demonstrating the species specificity of the designed primers (Fig. 2). Porcine CD86, CD54, CD58 and CD59 transcripts were detected in resting PAECs without detectable variation in the level of expression during the co-culture (Fig. 2). By contrast, CD80 transcripts were not detected in resting PAECs but were slightly induced after 24 h co-culture (Fig. 2). CD48 transcripts were not detected in PAECs, even during the co-culture.

Blocking experiments in the simplified direct model

The ability of the detected endothelial co-stimulation molecules to provide signals directly to human $CD4^+$ T lymphocytes was then studied in a simplified co-culture model in which highly purified huCD4$^+$ T lymphocytes were co-cultured for 3 days with PAECs in the presence of low doses of PHA. The human CTLA4-Fc fusion protein, known to bind porcine B7 molecules [25], did not affect the CD4$^+$ T lymphocyte proliferation (Fig. 3). Since this fusion protein, at identical concentrations,

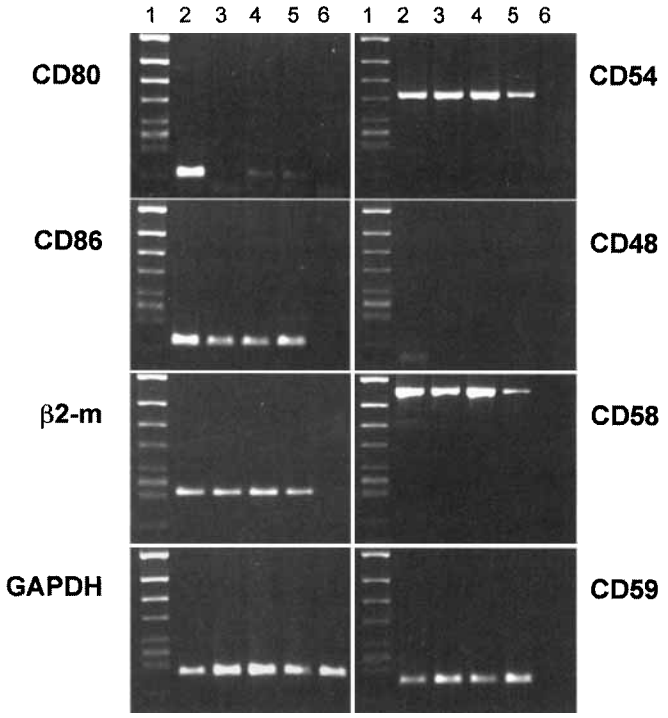

Fig. 2 Analysis of porcine co-stimulation molecule transcripts in porcine lymphoblastoid cell lines*2*; resting PAECs *3*; PAECs from 24 h XMLEC *4*; 72 h XMLEC *5*; human mature dendritic cells *6*. Molecular weight marker used is the ϕX174/HincII (Eurobio)

effectively blocked the proliferation of human T $CD4^+$ lymphocytes to porcine lymphoblastoid cell lines L23 and L35 [24], the lack of effect suggested that no B7 molecules were expressed on these porcine endothelial cells, which was surprising, if one takes into account the constitutive expression of CD86 transcripts (Fig. 2). The absence of B7 membrane expression on PAECs was, however, confirmed with flow cytometry experiments performed with human CTLA4-Fc fusion proteins from two different origins (Fig. 4).

The expression of two putative ligands of CD2 on resting PAECs, i.e. CD58 and CD59 (Fig. 2), prompted us to study the CD2 pathway by the blocking T11.2 mAb, which did not bind to porcine endothelial cells in flow cytometry experiments (data not shown). The 80% inhibition of the proliferation induced by this mAb (Fig. 3) indicates that the CD2 pathway is a major pathway of $CD4^+$ T lymphocyte co-stimulation in this simplified model. The CD54/LFA-1 pathway was analysed with a blocking anti-human CD11a monoclonal antibody that also does not recognise porcine endothelial cells (data not shown). This anti-human CD11a blocking mAb regularly inhibited lymphocyte proliferation by 30% in the simplified model (Fig. 3), indicating that the LFA-1 pathway is an additional putative way of human $CD4^+$ T lymphocyte co-stimulation triggered by PAECs.

Blocking experiments in the model of indirect presentation

When added in the classical XMLEC, the human CTLA4-Fc fusion protein inhibited proliferation by 83% (Fig. 5), which is in contrasts to the results obtained in the simplified model (Fig. 3). A similar blocking effect could also be demonstrated with a blocking anti-huCD28 mAb (Fig. 5). Considering the main involvement of the B7/CD28 pathway in the classical XMLEC model, we tested anti-human CD86 and anti-human CD80-blocking mAbs to identify the origin of the signals. Neither of these two mAbs recognised

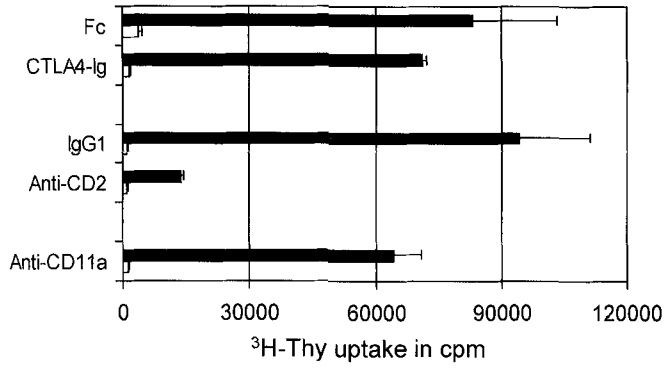

Fig. 3 Blocking experiments in the simplified co-culture model. Proliferative response of human $CD4^+$ T cells in response to PAECs with PHA (*dark columns*) and without PHA (*light columns*). Results are expressed in counts per minute (*cpm*), as the means ± SD of triplicate determinations. This value is derived from one experiment representative of five

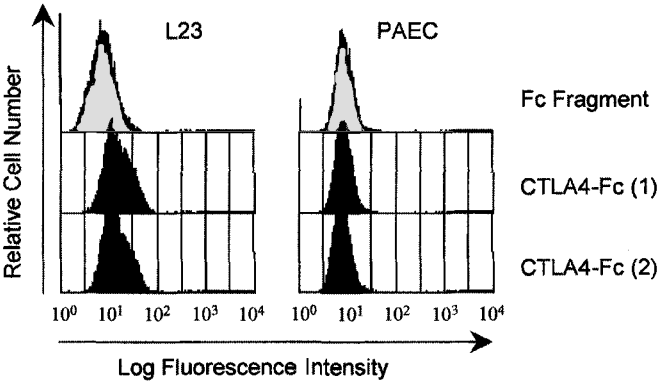

Fig. 4 Analysis of B7 molecule expression on PAECs by flow cytometry. We stained cells by indirect immunofluorescence, using human CTLA4-Ig fusion protein from either R&D Systems *1* or Bristol-Myers-Squibb *2*, or human Fc fragments as control. Control fluorescence was arbitrarily fixed at 10 on the logarithmic scale

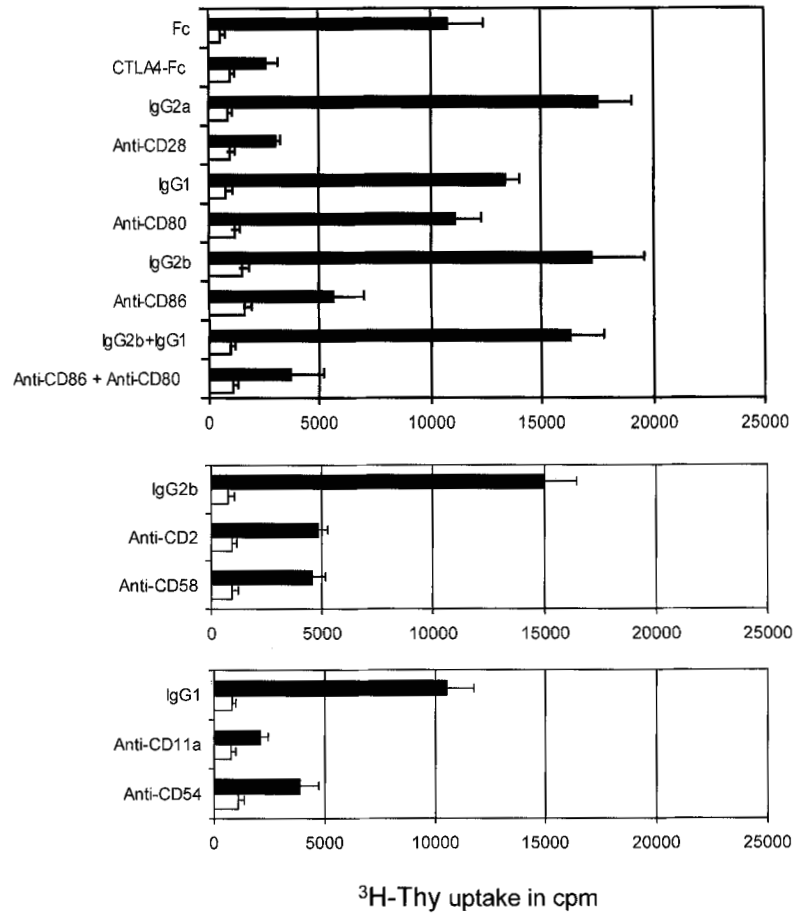

Fig. 5 Blocking experiments in the classical XMLEC model. *White column* human $CD4^+$ T cells alone; *black column* human $CD4^+$ T cells and human monocytes. Results are expressed in counts per min (*cpm*), as mean ± SD of triplicate determinations. This value is derived from one experiment representative of five

porcine endothelial cells or porcine cells expressing B7 molecules (data not shown). The anti-huCD86 mAb inhibited the $CD4^+$ T lymphocyte proliferation by 60%, and this inhibition was more pronounced when the anti-huCD80 mAbs were added. Altogether, the blocking effect of the anti-huCD86 and the anti-huCD80 mAbs was similar to the blockade induced by CTLA4-Ig or anti-huCD28 mAbs (Fig. 5), showing that the CD28 signal is due to the engagement of human B7 molecules.

We then studied the CD58/CD2 pathway, using the anti-human CD2 mAbs. As in the simplified model, the T11.2 mAb inhibited proliferation by 60% (Fig. 5). A similar inhibition was also obtained with an anti-huCD58 blocking mAb, which does not bind to PAECs (data not shown), again identifying the huAPC as the source of the CD2 co-stimulatory signal. A strong inhibition (65%) of the lymphocyte proliferation in the classical model was also obtained with the anti-huCD11a blocking mAb. As the level of inhibition produced by the anti-huCD54 blocking mAb was similar to the blockade produced by anti-huCD11a (Fig. 5), it was also concluded that the LFA-1 co-stimulatory signal originates from CD54 molecules expressed by the huAPCs.

Discussion

As a model entirely based on the indirect presentation pathway, the XMLEC provides a unique opportunity to discriminate between the lymphocyte co-signals provided by host APCs and those provided by graft-derived endothelial cells. The results presented here provide additional evidence that PAECs express co-stimulatory molecules and have the intrinsic ability to co-stimulate human $CD4^+$ T lymphocytes, particularly when both cells are forced to interact by PHA in a model mimicking the direct presentation. Nevertheless, we also show that all the co-stimulatory signals received by the $CD4^+$ T lymphocytes in the classical XMLEC model originate only from the APCs that deliver the cognate signal, i.e. the human monocytes [12]. No evidence of *trans*-co-stimulation by porcine endothelial bystander cells could be found.

The fact that PAECs can provide co-stimulatory signals to human T lymphocytes across the species barrier was, indeed, already known [13, 14, 15]. The confirmed importance of the CD2 pathway in the simplified model [13] is better explained by the endothelial

expression of CD58, which is demonstrated for the first time at the mRNA level. Indeed, PAECs do not express CD48 transcripts, and porcine CD59 seems unable to provide co-stimulation to human T cells [26], which provides evidence that the LFA-1 pathway is also probably linked to the expression of porcine CD54 by PAECs. This pathway has already been recognized [14] but not by all authors. The most surprising observation was the absence of human CTLA4-Fc binding to the PAECs, despite the presence of CD86 transcripts, and the failure of CTLA4-Fc to block the PHA-induced $CD4^+$ T cell proliferation. Because the same reagent detects B7 molecules on porcine lymphoblastoid cell lines, and could block the proliferative response induced by these cells [24], the CD86 transcript is probably not translated to protein, or the translated protein does not reach the cell membrane.

This is not the first time that an absence of B7 expression on PAECs has been observed [19], although several studies have identified the expression of CD86 on PAECs [27, 28]. To date we have found no explanation for these discrepancies. Despite the expression of co-stimulatory molecules by PAECs, the proliferation observed in the XMLEC appears to be based only on co–stimulatory signals delivered in the *cis*-configuration by the human monocytes. The three identified pathways (B7/CD28; CD58/CD2; CD54/LFA-1) are each of great importance because their individual blocking inhibits the proliferation in the range of 70%–90%, which indicates a lack of redundancy and the requirement of each pathway. The fact that *trans*-co-stimulation by the porcine ligands of huCD2 and huLFA-1 is not operating in the XMLEC could be explained by at least three non-mutually exclusive reasons. The first could be a better affinity or complementarity of the human LFA-1 and CD2 receptors for their respective human ligands rather than for their porcine ligands, despite the fact that pairing across the species barrier occurs in the simplified model. A second possible reason is the absence of B7 molecules on the endothelial cells. Indeed, in all the in vitro models where *trans*-co-stimulation was obtained [16, 17, 18], the co-stimulatory molecules acting in the *trans*-form were the B7 molecules. A third possible reason is a physiological preference for co-stimulation in the*cis*-form, when it exists, rather than in the *trans*-form. This fact has been demonstrated by the use of human fibroblaststransfected by major histocompatibility complex (MHC) class II molecules and/or by CD86, which induce a more significant proliferation when the expression of CD86 is in a *cis*-configuration [29]. Therefore, even in the presence of porcine B7 molecules on endothelial cells, it would be likely that the CD28 co-signals were provided in the *cis*-form by the huAPCs.

Finally, it appears that what we observed in vitro in a xenogeneic model is very similar to what was observed in vivo in an allogeneic model [19, 29]. Mandelbrot et al. have indeed shown that co-stimulation is always provided by the recipient APCs, in *trans*- when the allorecognition is direct and in *cis*- in the case of indirect allorecognition [29]. Our XMLEC model is, thus, of great significance for identifying the processes occurring at the vascular interface level to induce lymphocyte responses by the indirect presentation pathway. Since our data indicate that the active role played by endothelial cells in the XMLEC [12] does not provide co-stimulatory signals in *trans*-form, another explanation would be that endothelial cells favour xeno-antigen presentation and *cis*-co-stimulation by human monocytes. This could occur if xenogeneic endothelial cells were able to induce monocyte-to-dendritic cell differentiation and dendritic cell migration into secondary lymphoid organs, as allogeneic endothelial cells do [30]. In that case, human APCs would acquire the property to stimulate $CD45RA^+$ naive, besides $CD45RO^+$ memory, T cells. Experiments are currently in progress to study these possibilities.

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