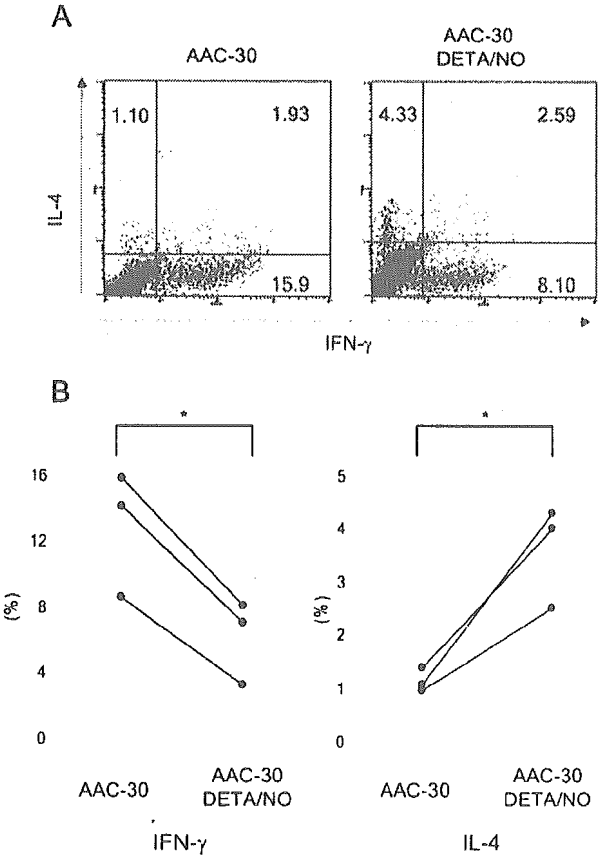

FIGURE 6. Effects of NO on polarization of naive CD4 T cells by pDCs. After pDCs were pretreated with AAC-30 (5 μM) in the absence or presence of DETA/NO (50 μM), they were irradiated and cocultured with allogeneic naive $CD4^+$ T cells for 7 days. T cells were internally stained for IFN-γ and IL-4 and analyzed with a FACScan. *A*, The data of one representative experiment are shown. The percentage of the individual cytokine producer T cells is indicated in each dot plot profile. *B*, The assay was repeated three times, and the percentage of the individual cytokine producer T cells is shown for each sample. The differences in both IFN-γ- and IL-4-producing cells were statistically analyzed by paired *t* test. *, $p < 0.05$.

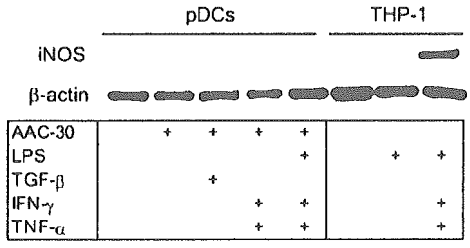

FIGURE 7. Effects of TLR ligands and cytokines on iNOS expression in pDCs and THP-1. pDCs and THP-1 were treated for 18 h in the absence or presence of the indicated TLR ligands: AAC-30 (5 μM) and LPS (1 μg/ml); and cytokines: TGF-β (10 ng/ml), IFN-γ (100 U/ml), and TNF-α (0.8 μg/ml). Expression of iNOS and β-actin, an internal protein control, was evaluated by Western blot analysis. The results shown are from one representative experiment of three consistent ones.

and/or LPS, whereas a subset of murine DCs express them (37–40). We examined whether treatment of human pDCs with TLR ligands such as AAC-30 and/or LPS together with several cytokines (32) induced expression of iNOS and subsequent generation of NO. In contrast to a human monocyte cell line, THP-1, that expressed iNOS upon stimulation with LPS and inflammatory cytokines as previously reported (41, 42), neither AAC-30 alone nor a combination of AAC30 and these inflammatory cytokines with or without LPS induced iNOS expression in pDCs (Fig. 7). Although Zhang et al. (43) have reported recently that TGF-β is involved in the differentiation of murine DCs into NO highly producing DCs, it had no effect on iNOS expression in human pDCs. In accordance with these results, we could not detect NO production of pDCs under these conditions with the Griess reaction (data not shown).

Discussion

In the present study, we showed that NO suppresses the production of several cytokines, especially IFN-α of activated pDCs partly via a cGMP-dependent pathway, up-regulates the OX40L expression on pDCs, and consequently polarizes them toward a Th2-promoting phenotype. We also demonstrated that NO inhibits the expression of IRF-7, which may be one of the possible molecular mechanisms by which the NO/cGMP system suppresses IFN-α production.

NO, a water- and lipid-soluble gas, is known to be critically involved in not only vascular relaxation but also immunological host defense (1–3). The main molecular target of NO eliciting most of its downstream effects is cytoplasmic soluble GC that catalyzes biosynthesis of intracellular cGMP (1, 4). NO is produced by three kinds of NO synthases. iNOS is one among them that produces a large amount of NO for a longer time (i.e., 10–100 times more) than neuronal NOS and endothelial NOS (3), and its expression is localized in the area of inflammatory lesion such as bacterial or viral infection (2, 3). In mice, bacterial infection, LPS, or inflammatory cytokines induce iNOS expression in DCs in vivo as well as in vitro (37, 38, 43). However, previous reports indicated that human myeloid DCs do not express iNOS by stimulation with LPS and IFN-γ (39, 40). In accordance with them, we could detect neither iNOS expression nor NO generation by pDCs stimulated with AAC-30, LPS, and several cytokines. Thus, NO-mediated regulation of human DCs is thought to depend on exogenous or paracrine NO, which is produced by activated macrophages as well as epithelial cells at inflammatory lesions (39).

IFN-α plays essential roles in antiviral innate immunity by directly inhibiting viral replication in infected cells and in immunoregulation by augmenting DC as well as NK cell function (20, 21). The expression of the *IFN-α* gene is largely regulated by a transcription factor, IRF-7 (29, 30). IFN-α is known to be produced by certain subsets of leukocytes and fibroblasts (21). Above all, pDCs produce a large amount of IFN-α in response to viral infection or CpG-oligonucleotides stimulation and consequently undergo differentiation into mature DCs (17–19, 44–46). Hence, they represent a unique cell lineage, which operates the two master functions of innate immunity and adaptive immune responses (22). In this context, NO/cGMP-mediated inhibition of IFN-α production by activated pDCs may have profound effects on the outcome of immune responses.

It is known that the balance of Th1/Th2 responses is influenced by the divergence of cytokines and costimulatory molecules of APCs (13, 14, 47). Activated pDCs produce high levels of IFN-α and consequently promote Th1 responses (19, 48). As in the case of histamine that has been reported to inhibit IFN-α production and impair the ability of pDCs to generate Th1 cells (49), NO did not alter the expression levels of CD80, CD86, and HLA-DR but suppressed IFN-α production. Furthermore, we observed that NO up-regulated the expression level of OX40L on AAC-30-stimulated pDCs. It should be noted that the OX40/OX40L system plays an important role in the formation of Th2 responses in both mice

and humans (33, 34). Recently, Ito et al. (50) have reported that OX40L exhibited costimulatory functions in human pDC-mediated Th2 responses. Based on these findings, polarization of pDCs toward a Th2-promoting phenotype by NO may be at least in part ascribed to the suppression of IFN-α production and up-regulation of OX40L expression.

Intracellular cGMP, which is not only generated by NO and its receptor soluble GC but also by natriuretic peptides and their receptors, regulates gene expression positively and negatively at transcriptional levels (4, 51). We previously reported that the receptor for atrial natriuretic peptide, GC-A, is expressed on monocyte-derived DCs and that atrial natriuretic peptide increases intracellular cGMP, inhibited LPS-induced IL-12 and TNF-α production, increased IL-10 production, and consequently polarized these DCs toward a Th2-promoting phenotype (52). Furthermore, our preliminary experiments showed that cGMP analogues suppressed IL-12 production by $CD11c^+$ myeloid blood DCs (data not shown). These findings may be explained partly by the reduction of NF-κB-binding activity by cGMP that several studies have already investigated in macrophages (51, 53). In the present study, we demonstrated for the first time that cGMP also down-regulated expression of IRF-7 in activated pDCs and consequently suppressed production of IFN-α, although the more detailed molecular mechanism of cGMP action is still unknown. Therefore, we hypothesize that a generalized scheme that cGMP functions as a common second messenger for the formation of Th2 responses in both myeloid and pDCs.

On the other hand, all the biological effects of NO are not mediated by cGMP (1, 3). In particular, evidence has indicated that relatively high concentrations of NO induce *S*-nitrosylation of certain molecules, resulting in their functional modulation. It has been reported that in murine macrophages, NO inhibits NF-κB and JNK1 activity by *S*-nitrosylation of these molecules (54, 55). Our data showed that suppression of IFN-α production of pDCs by DETA/NO was not completely reversed by an addition of ODQ, a specific inhibitor of soluble GC, suggesting that a cGMP-independent mechanism is also involved. It is to be determined if this cGMP-independent mechanism is mainly mediated by *S*-nitrosylation.

Previous studies with iNOS-deficient or -mutant mice have indicated that NO plays a critical role in the control of microbial pathogens, such as *Leishmania major*, *Mycobacterium tuberculosis*, and hepatitis B virus (6, 7, 56). Paradoxically, these mice can limit the pathological deteriorations caused by viruses such as coxsackievirus B3 and Sendai virus and their viral growth (10, 57). The iNOS-deficient mice infected with influenza A virus clear the virus from their lung and manifest less histopathologic changes in the lung than the wild-type mice (5, 57). With regard to these findings, it is suggested that the iNOS-deficient mice produce higher levels of IFN-γ and stronger Th1 responses than wild-type mice (5). In contrast, it is also relevant to our findings that blocking OX40/OX40L interaction has been reported to alleviate manifestations of influenza A virus-induced pneumonia as well as bronchial asthma (58, 59). Although the precise mechanism by which NO inhibits Th1 responses and deteriorates viral infection is unclear, it is likely that both events are ascribed in part to inhibition of IFN-α production and up-regulation of OX40L expression due to NO.

Both NO, especially excessively generated by iNOS, and IFN-α are thought to play an important role in pathophysiology of several allergic diseases. iNOS is found to be expressed in airway epithelial cells, macrophages, and eosinophils, and increased levels of exhaled NO have been detected in asthmatic and allergic rhinitis patients (23–25). NO not only causes oxidative tissue damage but also induces eosinophil migration and cascades of PGs and worsens asthma (2, 11, 12, 25, 60). Based on our results, NOS inhibitors not only reduce the tissue damages brought down by NO (60, 61) but also may be beneficial for the correction of Th2 dominant allergic reactions. In this context, it is noted that IFN-α production by PBMCs in ex vivo cultures is significantly lower in children with allergic than nonallergic diseases and that IFN-α administration has high efficacy in improving patients with severe asthma (62, 63). Furthermore, pDCs have been reported to accumulate in nasal mucosa of allergic subjects after allergen challenge, and adoptive transfer of pDCs inhibits development of asthma in a mouse model (27, 64). Although the precise mechanism of this phenomenon is unclear, it is suggested that IFN-α produced of these pDCs replenishes IFN-α levels lowered by NO generated at allergic lesion. Thus, NO and pDC-derived IFN-α may regulate the balance of Th1/Th2 immune responses.

In conclusion, the present study provides novel insights into the biological significance of NO and its second messenger cGMP in pDC-mediated regulation of Th1/Th2 responses. Further delineation of this aspect may lead to elucidation of the pathophysiology of allergic as well as infectious diseases and eventually to development of a novel therapy for them.

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Endothelial cell co-stimulation through OX40 augments and prolongs T cell cytokine synthesis by stabilization of cytokine mRNA

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Abstract

Human endothelial cells (ECs) constitutively express OX40L and co-stimulate memory CD4$^+$ T cell proliferation that is dependent upon OX40–OX40L interaction. *In vivo*, OX40 prolongs T cell survival; however, an unanswered question is whether it can also prolong synthesis of proliferation-sustaining cytokines such as IL-2. Here we show that EC co-stimulation results in the secretion of T cell IL-2, IL-3 and IFN-γ and that in the absence of OX40 signals synthesis largely ceases by 12–18 h, but is prolonged up to 60 h in the presence of OX40 signaling. Blocking OX40-mediated cytokine expression at later times suppresses T cell proliferation and this can be overcome by addition of exogenous IL-2. We find that OX40 signaling has discrete effects on T cell activation as it does not affect expression of IL-10, CD25, CD69 or soluble IL-2R. Also, OX40 does not appear to alter IL-2 transcription, but rather acts to stabilize a subset of cytokine mRNAs, increasing their half-lives by 3–6-fold. We further show that OX40L induces activation of p38 mitogen-activated protein kinase (MAPK) and phosphotidyl-inositol-3-kinase (PI3K) in T cells, and using specific inhibitors, we find that increased mRNA half-life is dependent upon both these pathways but is independent of c-jun-N-terminal kinase (JNK). Thus, EC co-stimulation through OX40 leads to prolonged T cell cytokine synthesis and enhanced proliferation.

Introduction

During immune responses in the periphery, vascular endothelial cells (ECs) play an important role in the recruitment and extravasation of leukocytes to sites of inflammation (1). Over the last decade, there has been accumulating evidence that human ECs also play an additional role during the immune response as antigen-presenting cells (APCs) and have the ability to activate both resting CD4$^+$ and CD8$^+$ memory T cells (1, 2). Activated human ECs express MHC class II molecules on their surface and provide the necessary co-stimulatory signals to fully activate memory CD4$^+$ T cells to secrete cytokines and proliferate (1–5). Studies in mice support an immunostimulatory role for EC where it appears vascular ECs are capable of presenting antigen (6) and directly activating CD8$^+$ T cells (7), but not CD4$^+$ T cells (8). Other differences between mouse and human may also limit the role of mouse ECs as APCs, and these preclude the use of mice as good models for antigen presentation by ECs (9). Despite this, some limited work has been performed in Hu-PBL-SCID mice, where allogeneic human ECs were found to be the major target for immune-mediated damage in transplanted human skin (10).

Human ECs do not express B7.1 or B7.2 and in the absence of CD28 ligands, they rely on alternative molecules to provide co-stimulation, such as inducible costimulator ligand (ICOSL) (M. Mazanet and C. Hughes, unpublished observations) (5), and CD58, which acts early to promote formation of the immune synapse through its interaction with CD2 (11). OX40 provides strong co-stimulatory signals to CD4$^+$ T cells and its ligand, OX40L, is expressed by human ECs (12, 13). Several other tumor necrosis factor (TNF) receptor family members, including 4-1BB (CD137), CD27, CD30, HVEM and OX40 (CD134), also provide co-stimulation to T cells and enhance effector function (14–20). However, EC expression of the ligands for these TNFR family members has not been reported.

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OX40 is inducibly expressed on T cells and cross-linking promotes cytokine synthesis, T cell proliferation and protection from apoptosis (21, 22). Recently, it was shown that OX40 acts through the PI3K pathway and targets protein kinase B (PKB; Akt). Blocking OX40 signals, or PKB activity, resulted in enhanced apoptosis and reduced T cell expansion (23). The effect of OX40 during a primary immune response is to expand the pool of effector cells and consequently the number of memory cells generated by suppressing apoptosis and prolonging proliferation (24). Not surprisingly, T cells lacking OX40, although capable of secreting IL-2 and proliferating normally during the initial period of activation, cannot sustain these processes during the later phases of the primary response. *In vivo* studies using OX40L knockout or transgenic mice support the idea that OX40 plays a role in regulating the persistence of an immune response (25–27).

OX40L is found on professional APCs, such as dendritic cells and activated B cells, and has recently been identified on human ECs, both *in vitro* and at sites of inflammation *in vivo* (12, 13). In this context, OX40–OX40L interaction may have a dual role as it mediates not only the adhesion of activated T cells to vascular ECs—and thus may play a role in the infiltration of activated T cells at sites of inflammation (12, 28)—but also provides co-stimulation, resulting in augmented IL-2 synthesis (19).

Although OX40 signaling is known to sustain T cell proliferation *in vivo* through increased cell survival, it has not been determined whether these late-acting signals can also prolong cytokine synthesis. This is an important question as adequate cytokine expression (especially IL-2) is essential for driving continued T cell proliferation. Here we show that OX40 signals do indeed prolong cytokine synthesis and do so through Akt/PKB and p38-mediated extension of cytokine mRNA half-life.

Methods

Antibodies and reagents

The superantigens (SAg), staphylococcal enterotoxin A, staphylococcal enterotoxin B and toxic shock syndrome toxin-1 (TSST-1) (Toxin Technology, Inc., Sarasota, FL, USA), were used in combination in all experiments. IFN-γ was from Biosource International (Camarillo, CA, USA). The following inhibitors were from Calbiochem (La Jolla, CA, USA)—target enzyme and final concentration are shown in parentheses: SB203580 (p38 MAPK, 10 μM); LY294002 (PI3K, 10 μM); SP600125 (JNK1, 2 and 3, 5 μM). Dimethyl sulfoxide was used as diluent and control. Control antibody (HB64) and anti-CD2 mAb (TS2/18) were purified from hybridoma cells purchased from American Type Tissue Collection (Rockville, MD, USA). FITC-conjugated anti-OX40 mAb was from Pharmingen (San Diego, CA, USA).

Cell purification and culture

PBMCs and human umbilical vein endothelial cells (HUVEC) were isolated as previously described (11). $CD4^+$ T cells were purified from PBMCs using MACS $CD4^+$ T cell Isolation Kits (Miltenyi Biotec, Auburn, CA, USA). In experiments using SAg, ECs were used at passage 2–4 and treated with 25 ng ml^{-1} IFN-γ for 3 days to induce the expression of MHC class II.

T cell cytokine secretion

$CD4^+$ T cells were plated on a monolayer of MHC II^+ ECs in 24-well culture plates. The T cells were stimulated with 0.5 ng ml^{-1} SAg in the presence of either 5 μg ml^{-1} anti-OX40L mAb or 5 μg ml^{-1} HB64 control mAb. Supernatants were assayed for the accumulation of IL-2, IL-3, IL-10, IFN-γ and soluble IL-2R using the DuoSet ELISA Development System (R&D Systems, Minneapolis, MN, USA). Protein secretion was also measured in discrete windows of time by harvesting supernatants every 12 h and replacing with fresh media containing SAg and either anti-OX40L mAb (ik-5) or HB64 mAb. Expression of CD25 and CD69 in T cells was also measured throughout the time course by FACS analysis.

T cell proliferation

$CD4^+$ T cells were plated on a monolayer of class II^+ ECs, pretreated with 100 μg ml^{-1} mitomycin C for 30 min at 37°C in 96-well culture plates. The T cells were stimulated with 0.5 ng ml^{-1} SAg in the presence of either 10 μg ml^{-1} anti-OX40L mAb or 10 μg ml^{-1} HB64 mAb. Where indicated, the supernatants were removed at 24 h and replaced with fresh media containing SAg and mAb. IL-2 was added, where indicated, at 50 U ml^{-1}. Cells were pulsed with 1 μCi [^{3}H]thymidine at 80 h, harvested at 96 h and analyzed for thymidine incorporation.

T cell co-stimulation assays using 293-OX40L stable cell line

$CD4^+$ T cells were spun down onto anti-CD3 mAb (64.1)-coated plates, followed by addition of 293 cells expressing OX40L or B7.2 or vector-transfected control cells. Supernatants were collected at various times thereafter for the measurement of cytokine secretion by ELISA. Luciferase assays were performed by transfecting $CD4^+$ T cells with 60 μg ml^{-1} of either an IL-2 promoter–luciferase construct containing 600 bp genomic human IL-2 sequence 5′ of the start site (−600IL-2-luc) or genomic murine IL-2 sequence from −2100 bp 5′ of the start site to +3000 bp (−2100IL-2-luc—a kind gift of Susan Ward, California Institute of Technology, USA). Transfections were performed using the Amaxa Nucleofection system (Amaxa, Köln, Germany) prior to anti-CD3 stimulation.

Quantitative reverse transcription–PCR analysis and cytokine mRNA half-life determination

T cell cytokine mRNA levels were measured at 24 h by quantitative reverse transcription–PCR, using cyber green for product detection, exactly as described (29). To determine cytokine mRNA half-lives, cultures were treated with signal pathway inhibitors as indicated and with 10 μg ml^{-1} actinomycin D (ActD) at 15 h and harvested 0, 1, 2 and 3 h later. Half-lives were calculated from linear regression best-fit semi-log plots of the log of the concentration of RNA versus time of culture with ActD. The $t_{1/2}$ values were calculated at either 2 or 3 h post-ActD and were derived according to the

equation $t_{1/2} = \ln 2/k_{decay}$, where $k_{decay} = (-2.3) \times$ the slope of the linear portion of the semi-log plots for each gene.

Western blotting

Purified $CD4^+$ T cells were cultured for 48 h with immobilized anti-CD3 and soluble anti-CD28 mAbs to induce OX40 expression. Approximately 75% of cells are induced to express OX40 under these conditions. Cells were then washed and rested in RPMI + 0.25% human serum albumin for 1 h, removed from the wells and re-plated onto BSA- or megaOX40L-coated wells. Plates were then briefly centrifuged to settle the cells. MegaOX40L is a trimerized form of OX40L (Alexis Biochemicals). Where indicated, cells were incubated with LY294002 beginning 15 min before addition to megaOX40L-coated plates. Cells were harvested after 30 min and western blotting was performed using antibodies to phospho-p38, total p38, phospho-Akt (all from Cell Signaling) and total Akt (Santa Cruz).

Results

OX40 signaling prolongs cytokine expression by $CD4^+$ T cells

We have shown previously that blocking CD2–CD58 interaction inhibits IL-2 synthesis during the initial phase of T cell activation by ECs (11). It is possible that IL-2 secretion in the later stages of T cell activation is, in part, mediated through the induction of other co-stimulatory molecules, such as OX40. To examine whether OX40 plays such a role, we first determined the temporal expression profiles of OX40 and OX40L on $CD4^+$ T cells and ECs, respectively. OX40 expression was low or absent on resting $CD4^+$ T cells but was detectable by 12 h and was peaking at ~48 h (Fig. 1A and B). In contrast, OX40L expression on ECs was found to be constitutive *in vitro* and did not change over time, although it can be up-regulated by exogenously added TNF and IFN-γ (D. A. Johnston and C. C. W. Hughes, unpublished observations). To determine the effects of blocking OX40 signaling on T cell IL-2 secretion during the late stages of T cell activation by ECs, we stimulated $CD4^+$ T cells with SAg and over time measured IL-2 secretion in the presence of either blocking antibodies to CD2 or OX40L or both (Fig. 1C). EC co-stimulation induced T cell IL-2 secretion by 6 h and this was almost completely blocked by disrupting CD2–CD58 interaction. However, disruption of OX40–OX40L interaction did not inhibit IL-2 secretion at this time, consistent with the lack of significant OX40 expression before 12 h. In agreement with our previous studies, IL-2 secretion was only partially blocked by anti-CD2 mAbs at 24 h. At the same time point, blocking OX40 signaling reduced IL-2 synthesis by approximately the same amount, 30–40%. The combination of anti-CD2 and anti-OX40L mAbs had an additive effect—blocking the effects of EC co-stimulation on T cell IL-2 secretion by ~60% at 24 h. TCR stimulation using anti-CD3 mAb or PHA in the presence of ECs produced similar results to those seen with SAg (data not shown). These data suggest that CD2 ligation is critical for the early stages of T cell activation by ECs and that OX40 signaling is important in sustaining IL-2 synthesis later in the process.

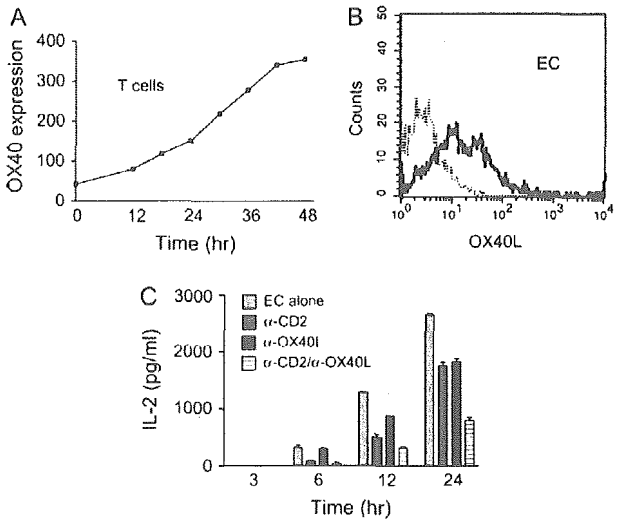


Fig. 1. Kinetics of OX40 and OX40L expression on $CD4^+$ T cells and ECs. T cells were plated on ECs pre-treated with IFN-γ to induce MHC class II expression, in the presence of SAg (0.5 ng ml^{-1}) and were incubated for 48 h at 37°C. (A) OX40 expression was measured on $CD4^+$ T cells and is shown as corrected mean fluorescence intensity. (B) OX40L expression on resting $CD31^+$ EC was analyzed by FACS. Solid and dotted lines represent ECs stained with anti-OX40L and control mAb, respectively. (C) Inhibition of EC co-stimulation by blocking OX40–OX40L interactions. $CD4^+$ T cells and ECs were cultured, as above, in the presence of either 10 μg ml^{-1} anti-CD2 mAb (TS2/18) or 5 μg ml^{-1} anti-OX40L mAb (ik-5) or both. The secretion of IL-2 into culture supernatants was measured over time by ELISA. Shown are means ± SEM from triplicate samples. One of two similar experiments.

We next examined the effects of EC-mediated OX40 signaling on secretion of other T cell cytokines. EC co-stimulation induced the synthesis of T cell IL-2, IL-3, IFN-γ and IL-10, and in the presence of anti-OX40L blocking mAb, secretion of IL-2, IL-3 and IFN-γ was significantly inhibited (Fig. 2A). IL-10 expression, however, was not affected by OX40L mAb. These data indicate that OX40 signals augment the synthesis of T cell IL-2, IL-3 and IFN-γ but not the synthesis of IL-10.

A drawback to measuring cytokine production *in vitro* is that it results in a misleading accumulation of cytokine over time, which sharply contrasts cytokine production *in vivo*, where cytokines are secreted and diffuse or are rapidly washed away. To determine the expression profile of OX40-mediated T cell cytokine synthesis within discrete windows of time, we stimulated $CD4^+$ T cells, once again, in the presence or absence of blocking antibodies to OX40L and measured IL-2, IL-3, IL-10 and IFN-γ secreted within consecutive 12-h-windows of time (Fig. 2B). Within the first 12 h of T cell activation, there was no significant difference in the quantity of cytokines produced in the presence or absence of an anti-OX40L mAb, consistent with the expression kinetics of OX40 on $CD4^+$ T cells (Fig. 1A). During the 12–24, 24–36 and 36–48 h-windows of time, disruption of OX40–OX40L interaction caused a dramatic decrease in the amount of IL-2, IL-3 and IFN-γ produced compared with control. For example, IFN-γ

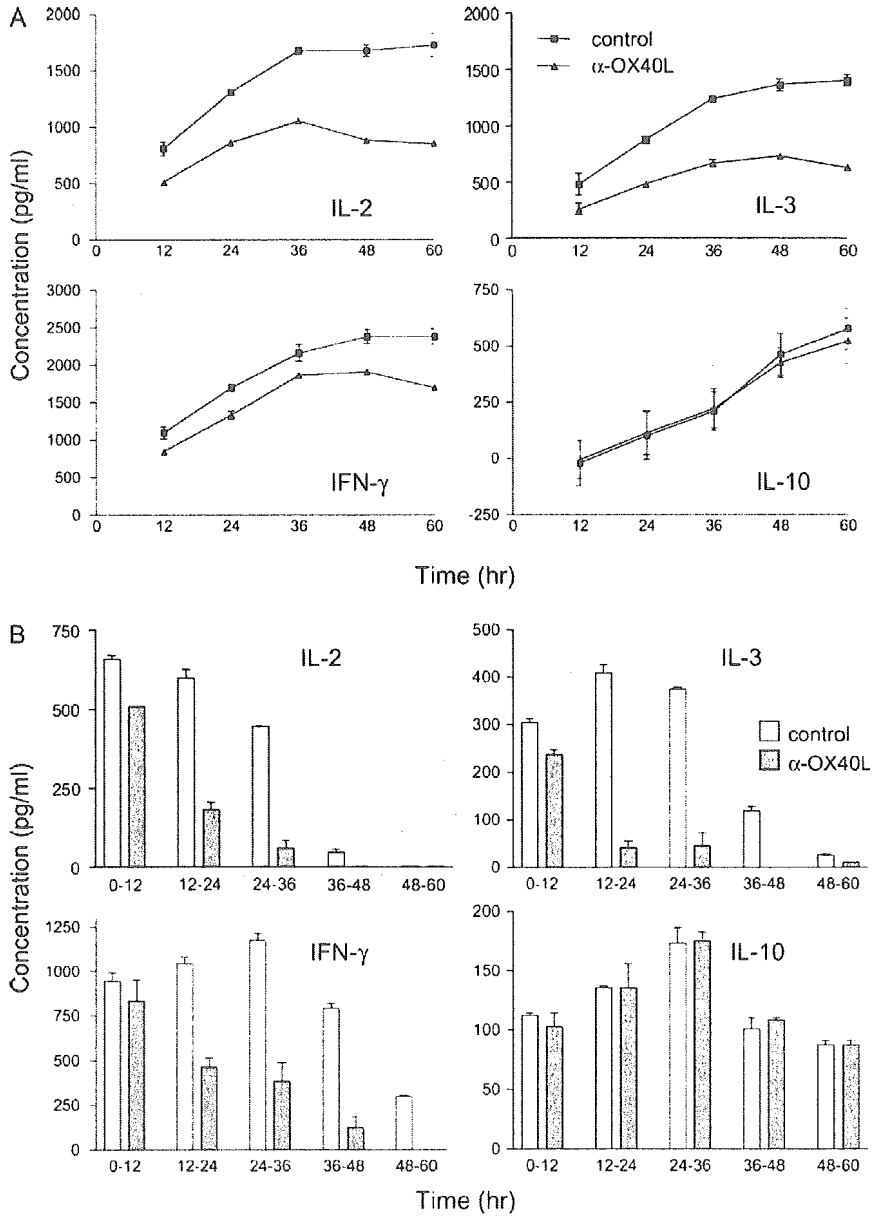


Fig. 2. Inhibition of T cell cytokine synthesis by blocking OX40 signaling. (A) T cells were plated on ECs pre-treated with IFN-γ to induce MHC class II expression, in the presence of SAg (0.5 ng ml^{-1}) and either 5 μg ml^{-1} anti-OX40L mAb (ik-5) or control antibody (HB64) and were incubated for 60 h at 37°C. (A) Culture supernatants were harvested at the indicated times and measured for the presence of IL-2, IL-3, IL-10 and IFN-γ by ELISA. Shown are means ± SEM from triplicate samples. One of three similar experiments. (B) Culture supernatants were harvested every 12 h and replaced with fresh media containing SAg and either anti-OX40L or control antibody. Supernatants were measured for the presence of IL-2, IL-3, IL-10 and IFN-γ by ELISA. Shown are means ± SEM from triplicate samples. One of three similar experiments.

was inhibited by >50% between 12 and 24 h and by >80% between 36 and 48 h. Inhibition of OX40 signaling did not affect IL-10 synthesis. These data suggest that OX40–OX40L interaction prolongs the synthesis of T cell IL-2, IL-3 and IFN-γ expression during T cell activation by human vascular ECs.

To determine whether the lower levels of cytokine had functional consequences we measured T cell proliferation in similar assays. In agreement with previous studies (22, 30), inhibition of OX40 signaling significantly blocked T cell proliferation, particularly if the medium was changed after 24 h, thus washing out previously synthesized cytokines (Fig. 3). Moreover, this block could be overcome by addition of IL-2, confirming the importance of OX40-mediated IL-2 expression in driving T cell proliferation. Interestingly, the expression of activation markers, such as CD25, CD69 and soluble IL-2R, was not affected by disrupting OX40–OX40L interaction

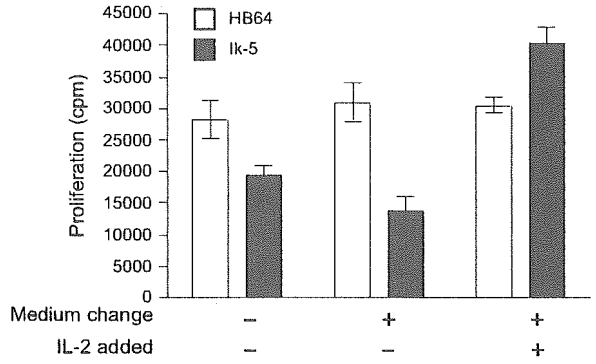


Fig. 3. OX40 signaling promotes T cell proliferation. T cells were plated on mitomycin C-treated MHC class II$^+$ ECs in the presence of SAg (0.5 ng ml^{-1}) and either 10 μg ml^{-1} anti-OX40L mAb (ik-5) or control antibody (HB64). Where indicated the supernatants were removed at 24 h and replaced with fresh media, containing SAg and ik-5 mAb or HB64 mAb. IL-2 was added, where indicated, at 50 U ml^{-1}. Cells were pulsed with 1 μCi [^{3}H]thymidine at 80 h and harvested at 96 h for analysis of thymidine incorporation. Shown are means ± SEM from triplicate samples. One of two similar experiments.

(Fig. 4), suggesting that OX40 signaling does not globally affect T cell activation events.

OX40L-expressing 293 cells co-stimulate T cell activation

Using anti-OX40L blocking mAb, we demonstrated that OX40 signaling was necessary for driving T cell activation events such as cytokine synthesis and proliferation. We next tested whether OX40L is sufficient to provide a co-stimulatory signal to T cells resulting in prolonged cytokine synthesis. An OX40L-expressing stable cell line (293^{OX40L}) and a control cell line (293^{vector}) were generated and several clones were isolated and analyzed for OX40L expression. Figure 5A illustrates the OX40L expression profile of clone #4, which was used in all further experiments. OX40L was expressed at higher levels than those observed on ECs and expression levels did not decrease with subsequent cell passage. Vector-transfected 293 cells did not express OX40L (Fig. 5A).

To assess the efficacy of 293^{OX40L} cells in providing co-stimulation, we stimulated CD4$^+$ T cells with plate-bound anti-CD3 mAb in the presence of either 293^{OX40L} or 293^{vector} cells and analyzed supernatants for IL-2 secretion after 24 h. In the presence of 293^{vector} cells, TCR stimulation induced IL-2 secretion, and the addition of anti-OX40L blocking mAb had no significant effect on cytokine synthesis (Fig. 5B). However, in the presence of 293^{OX40L} cells, IL-2 secretion was augmented ~3-fold and disruption of OX40–OX40L interaction completely blocked this effect. We next examined the ability of 293^{OX40L} cells to provide co-stimulation to T cells in comparison with human ECs. Dose–response curves were generated for T cell activation by plate-bound anti-CD3 mAb or SAg using 293^{OX40L} cells or ECs, respectively (Fig. 5C). Again, 293^{OX40L} cells provided a strong co-stimulatory signal and the combination of 293^{OX40L} cells and anti-CD3 mAb induced a similar magnitude of IL-2 secretion, as did EC–SAg. Thus, OX40 signaling is sufficient to provide co-stimulation to T cells, and 293^{OX40L} cells provide a useful tool to isolate the co-stimulatory actions of

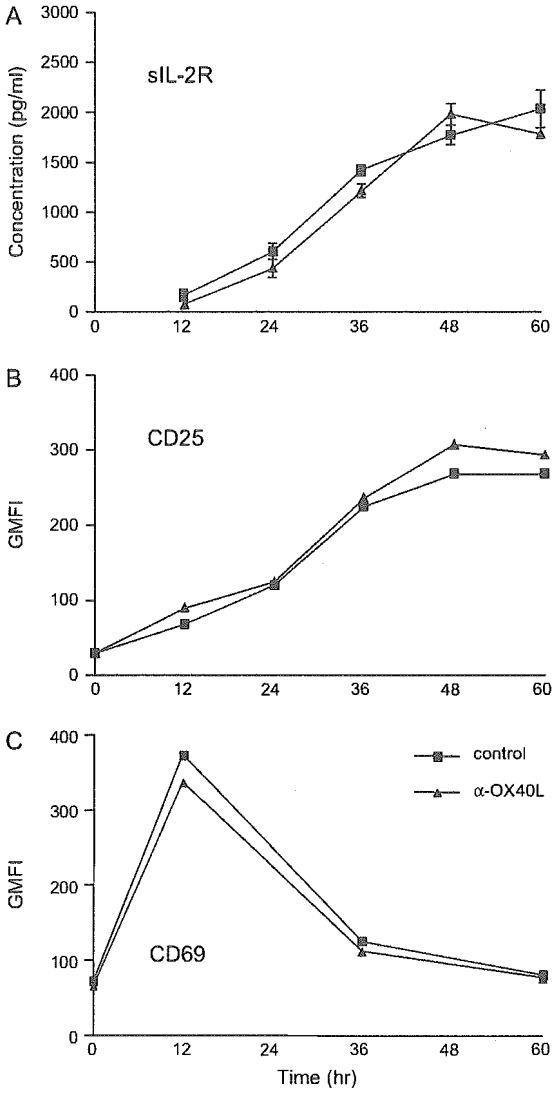


Fig. 4. The effects of blocking OX40 signaling on T cell activation marker expression. T cells were plated on ECs pre-treated with IFN-γ to induce MHC class II expression, in the presence of SAg (0.5 ng ml^{-1}) and either 5 μg ml^{-1} anti-OX40L mAb (ik-5) or control antibody (HB64) and were incubated for 60 h at 37°C. (A) Culture supernatants were harvested every 12 h and measured for the presence of soluble IL-2R by ELISA. Shown are means ± SEM from duplicate samples. (B, C) Cells were harvested every 12 h and stained with PE–CD4 and FITC–CD25 or FITC–CD69 mAbs. Cells expressing CD4 were analyzed for CD25 and CD69 expression by FACS. One of two similar experiments.

OX40L from those of other molecules found on ECs such as ICOSL.

OX40 signaling augments T cell cytokine mRNA levels

Using the 293^{OX40L} cells, we investigated the mechanism by which OX40-mediated signaling leads to the augmentation and prolongation of T cell cytokine synthesis. It was important to use the 293^{OX40L} cells rather than ECs as this ensured minimal interference from other co-stimulatory molecules

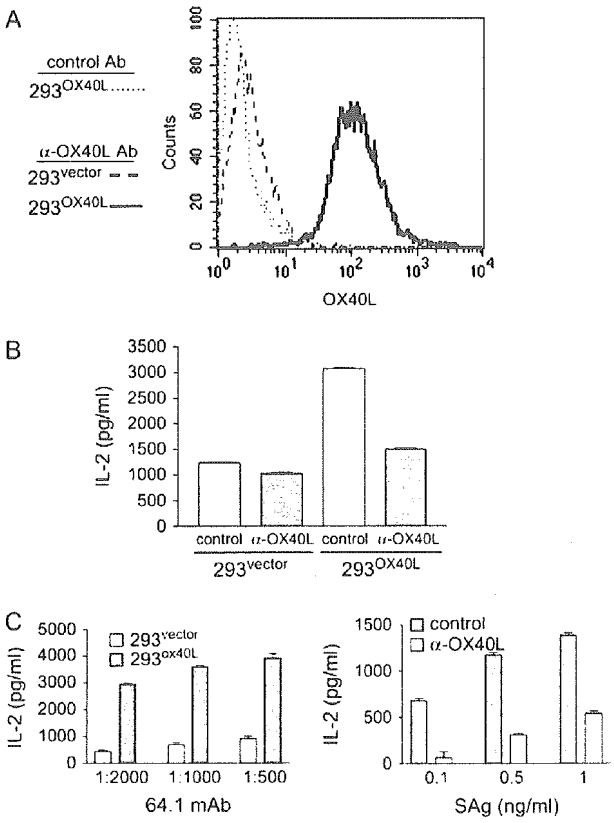


Fig. 5. OX40L-expressing human embryonic kidney (HEK) 293 cells co-stimulate T cell activation. HEK 293 cells were stably transfected with pcDNA3.1-OX40L expression vector (293^{OX40L}) or empty pcDNA3.1 vector (293^{vector}). (A) Clones were then analyzed for OX40L expression by FACS analysis. Shown are 293^{OX40L} stained with control antibody (dotted line), 293^{vector} stained with OX40L antibody (dashed line) and 293^{OX40L} stained with OX40L antibody (solid line). (B) $CD4^+$ T cells were stimulated with plate-bound anti-CD3 mAb (64.1) in the presence of either 293^{vector} or 293^{OX40L} and either anti-OX40L mAb or control antibody for 24 h. IL-2 secretion was measured from culture supernatants by ELISA. Shown are means ± SEM from triplicate samples. One of two similar experiments. (C) $CD4^+$ T cells were stimulated with various concentrations of plate-bound anti-CD3 mAb (64.1) in the presence of 293^{vector} 293^{OX40L} cells for 36 h (left). As a control, $CD4^+$ T cells were stimulated with ECs and SAg as before in the presence of control or anti-OX40L mAb for 36 h (right). IL-2 secretion was measured from culture supernatants by ELISA. Shown are means ± SEM from triplicate samples. One of two similar experiments.

present on the ECs. We began by examining the time course of cytokine mRNA expression. $CD4^+$ T cells were stimulated with plate-bound anti-CD3 mAb in the presence of either 293^{OX40L} or 293^{vector} cells and then harvested at various times over a 24-h period. Consistent with the absence of OX40 on activated T cells at early times, IL-2 mRNA levels were comparable at 6 h in the presence or absence of stimulation by OX40L (Fig. 6A). In the absence of OX40 stimulation, IL-2 mRNA levels then decreased over time. OX40 stimulation, however, resulted in elevation of mRNA levels beginning at 6 and continuing up to 24 h. Similar results were obtained for

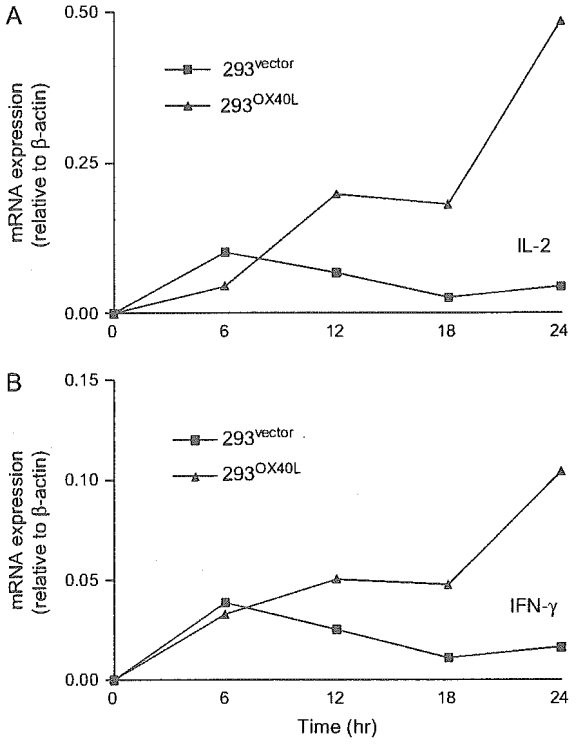


Fig. 6. OX40-mediated T cell cytokine mRNA expression. $CD4^+$ T cells were stimulated with plate-bound anti-CD3 mAb (64.1) in the presence of either 293^{vector} or 293^{OX40L} for 24 h. Cells were harvested every 6 h and total RNA was isolated for cytokine mRNA measurement by reverse transcription–PCR. (A) Expression of IL-2 and (B) IFN-γ relative to β-actin mRNA. One of two similar experiments.

IFN-γ mRNA expression (Fig. 6B). These data support our previous results, which showed that OX40 signaling augments T cell cytokine synthesis and illustrate the importance of OX40 signaling in maintaining high cytokine message levels late in the activation process.

Nuclear run-on analysis and use of IL-2 promoter–reporter constructs have shown that in the presence of EC co-stimulation the transcriptional rate of IL-2 is augmented within the first 6 h of activation but then falls so that transcription is largely arrested by 12 h (31). To examine whether OX40 signaling triggers a new wave of IL-2 transcription later in T cell activation, we transfected $CD4^+$ T cells with a luciferase reporter construct containing 600 bp upstream of the IL-2 transcription start site (−600IL-2-luc) and stimulated them with PHA in the presence of either 293^{OX40L} or 293^{vector} cells (Fig. 7A). OX40 stimulation had no significant effect on the transcriptional activity of the IL-2 promoter—luciferase activity was high at 6 h and decreased between 6 and 24 h, indicating that promoter activity was minimal at these later times. We were concerned that important regulatory elements may be missing from this short promoter and so we repeated the experiments using a reporter containing 2 kb of upstream sequence and 3 kb of downstream DNA (−2100IL-2-luc). This reporter was also unresponsive to OX40 signals (Fig. 7B), although interestingly, luciferase activity did not decrease over

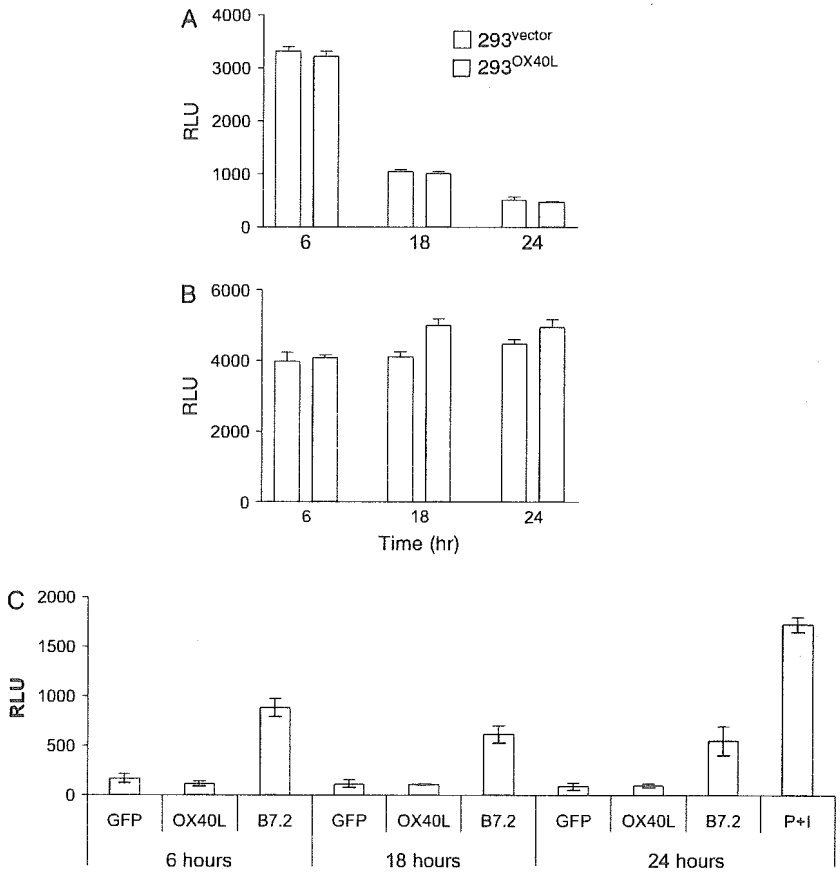


Fig. 7. Effects of OX40 signaling on T cell cytokine gene transcription. $CD4^+$ T cells were transfected with (A) −600IL-2-luc or (B) −2100IL-2-luc and stimulated with 5 μg ml^{-1} PHA in the presence of either 293^{vector} or 293^{OX40L} for 24 h. Cells were harvested at various times and analyzed for luciferase activity. Shown are means ± SEM from triplicate samples. One of two similar experiments. (C) T cells were transfected as above with −600IL-2-luc and cultured in the presence of 293^{vector}, 293^{OX40L} or $293^{B7.2}$ for 24 h. Shown are means ± SEM from triplicate samples. One of two similar experiments.

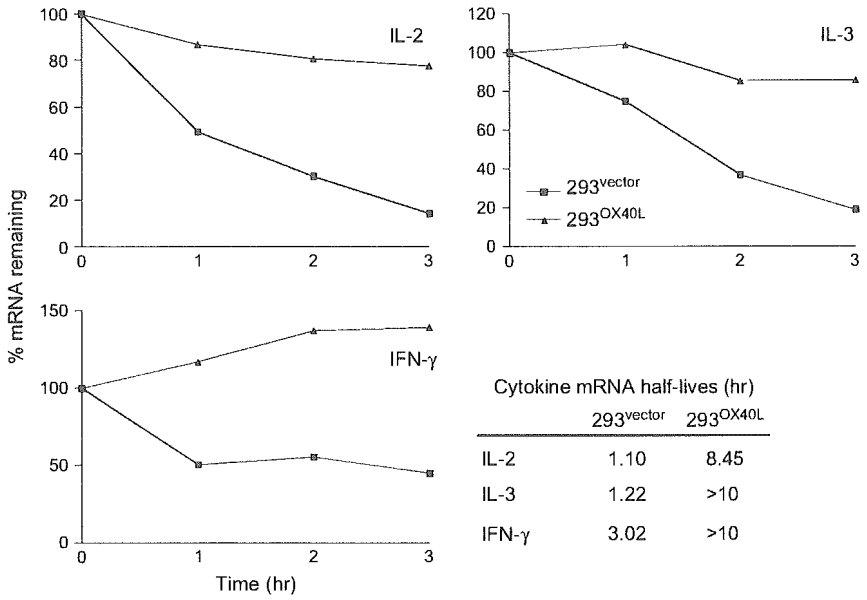
time, suggestive of ongoing, OX40-independent transcriptional activity dependent upon sequences outside of the 600-bp minimal promoter. We confirmed that these reporters were responsive to co-stimulatory signals by culturing transfected cells with 293 cells expressing B7.2. Both the short and the long forms were strongly induced by B7.2 but not by OX40L (Fig. 7C and data not shown). Together, these data suggest that OX40 signaling does not regulate T cell IL-2 transcription.

OX40 signaling stabilizes T cell cytokine mRNA

A second mechanism by which the steady-state levels of mRNA can be increased is by the stabilization of mRNA transcripts. To determine whether OX40 signaling affects the stability of T cell IL-2, IL-3 and IFN-γ mRNA, we activated $CD4^+$ T cells in the presence of either 293^{OX40L} or 293^{vector} cells (Fig. 8). Cells were treated with the transcriptional inhibitor, ActD, at 15 h and harvested for RNA analysis every hour thereafter for 3 h. In the absence of OX40 signaling, the message levels of IL-2, IL-3 and IFN-γ decreased rather quickly, with half-lives ranging from 1 to 3 h. Remarkably, however, OX40 stimulation increased the half-lives of IL-2, IL-3 and IFN-γ mRNA up to 10-fold (Fig. 8). We saw no effect of OX40L on IL-10 mRNA stability (data not shown). These data clearly demonstrate that OX40 signaling regulates the synthesis of T cell IL-2, IL-3 and IFN-γ through the stabilization of mRNA transcripts.

OX40-mediated T cell cytokine message stabilization is dependent upon the p38 MAPK and PI3K-Akt/PKB pathways

The stability of mRNA transcripts is regulated via RNA-binding proteins such as HuA, BRF1 and tristetraprolin (32, 33) binding to AU-rich elements (AREs) in the 3′ untranslated region (UTR) of many genes, including the cytokines IL-2, IL-3 and IFN-γ. Moreover, in different cell types, this process has been shown to be downstream of PI3K (34), p38 MAPK (35) and JNK (36). To examine the role of these various pathways in OX40-mediated stabilization of T cell cytokine mRNA, we activated $CD4^+$ T cells in the presence of either 293^{OX40L} or 293^{vector} cells



Cytokine mRNA half-lives (hr)

	293^{vector}	293^{OX40L}
IL-2	1.10	8.45
IL-3	1.22	>10
IFN-γ	3.02	>10

Fig. 8. Effects of OX40 signaling on T cell cytokine message stability. $CD4^+$ T cells were stimulated with 5 μg ml^{-1} PHA in the presence of either 293^{vector} or 293^{OX40L}. Cells were treated with 10 μg ml^{-1} ActD at 15 h and harvested at 15, 16, 17 and 18 h. Total RNA was isolated for cytokine mRNA measurement by reverse transcription–PCR. Shown is the percentage of mRNA expression relative to the 15 h (t = 0) time point. mRNA half-lives were calculated as described in Methods. One of three similar experiments.

Table 1. Involvement of the p38 and PI3K-Akt pathways in OX40-mediated T cell cytokine mRNA stabilization[a]

	293^{vector}	293^{OX40L}	293^{OX40L} + Inh.	% Decrease	Number of experiments
p38 Inh.					
IL-2	0.98	4.18	1.37	88 ± 7.5	5
IFN-γ	1.85	4.96	2.56	58 ± 6.3	5
PI3K Inh.					
IL-2	0.75	2.41	0.83	85 ± 10.5	4
IFN-γ	0.74	2.19	2.02	18 ± 18	2

[a]Shown are the mean half-lives of mRNA (hour) in the presence of control, OX40L or OX40L + inhibitor, the % decrease in half-life in the presence of drug (mean ± SEM) and the number of experiments. $CD4^+$ T cells were stimulated with 5 μg ml^{-1} PHA in the presence of either 293^{vector} or 293^{OX40L} cells. Cultures were treated with inhibitors and 10 μg ml^{-1} ActD and harvested 0, 1, 2 and 3 h later. Total RNA was isolated for cytokine mRNA measurement by quantitative reverse transcription–PCR. Inh., inhibitor.

and treated cultures with drugs just prior to treatment with ActD. Consistently, we found that inhibitors to p38 MAPK (SB203580) and PI3K (LY294002) significantly blocked OX40L-induced mRNA stabilization (Table 1). The PI3K inhibitor reduced IL-2 mRNA half-life in the presence of OX40L up to 85%, while, in contrast, the same inhibitor only had a mild effect (0–36% inhibition) on IFN-γ mRNA. The p38 inhibitor (which also has effects on JNK, see below), on the other hand, effectively reduced the mRNA half-life of both IL-2 (88% decrease) and IFN-γ (58% decrease) in addition to also reducing IL-3 mRNA half-life (>80%, data not shown). Interestingly, an inhibitor of JNK1, 2 and 3 (SP600125) had no effect on stability of either IL-2 or IFN-γ in OX40L-stimulated cells (data not shown), confirming that our results with the 'p38 inhibitor' SB203580 were reflective of the inhibition of p38 and not collateral inhibition of JNKs (37).

To determine whether p38 and PI3K are direct targets of OX40 signaling in human T cells, we cultured purified $OX40^+$ $CD4^+$ T cells with recombinant OX40L and looked for phosphorylation of p38 and the PI3K target Akt/PKB. As shown in Fig. 9, OX40 stimulation augmented phosphorylation of p38 several fold, comparable to the effect of serum. Akt/PKB phosphorylation was also augmented and this was blocked by LY294002. These data indicate that both the p38 MAPK and the PI3K-Akt/PKB pathways are activated downstream of OX40. In aggregate, our data suggest that at least two pathways downstream of OX40, namely p38 MAPK and PI3K-Akt/PKB, are involved in stabilizing a subset of cytokine mRNAs.

Discussion

Our results demonstrate that OX40 signaling in T cells, as a result of interaction with OX40L-expressing ECs, acts to stabilize a subset of cytokine mRNAs through p38 MAPK- and PI3K-dependent mechanisms. As a consequence of enhanced mRNA stability, cytokine synthesis is increased and this drives T cell proliferation. Our results suggest that human ECs in the periphery may help to sustain local inflammatory responses by prolonging cytokine expression.

Co-stimulation of memory T cells by human ECs may be a fundamentally distinct process in the early hours of EC–T cell contact compared with later times. Early signals through CD2 result in the augmentation of T cell IL-2 transcription and

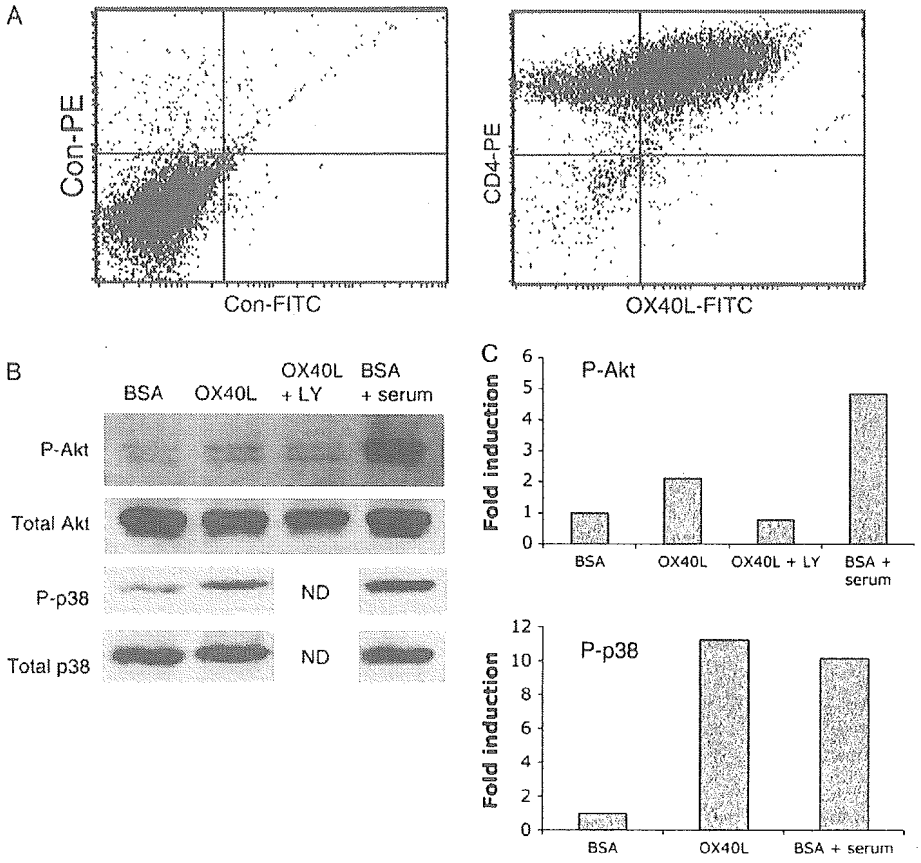


Fig. 9. OX40 signaling targets p38 and PI3K in T cells. (A) $CD4^+$ T cells were activated for 48 h with anti-CD3 and anti-CD28 mAbs to induce OX40 expression. (B) OX40-expressing cells were rested and then stimulated for 30 min with recombinant OX40L, in the presence or absence of LY294002 or serum as indicated. Lysates were analyzed by western blotting. (C) Western blots were quantitated and phosphorylated protein was normalized to total protein. One of three similar experiments.

protein synthesis as a result of augmented immune synapse formation (11). Disruption of CD2–CD58 interaction completely blocks IL-2 synthesis at early time points (<6 h) but only partially at late times (Fig. 1C), likely as a result of compromised immune synapse formation during the initial EC–T cell contact phase. In contrast, blocking OX40–OX40L interaction does not affect early IL-2 synthesis but significantly inhibits its synthesis at later times (Fig. 1C), in agreement with the delayed onset of OX40 expression on activated T cells (Fig. 1A). Thus, in addition to enhancing T cell survival through induction of the anti-apoptosis genes Bcl-xL and Bcl-2 (22, 23), our results show that OX40 also enhances T cell survival and proliferation through augmented cytokine synthesis late in the response. Moreover, OX40 acts later than CD2 and by a different mechanism. Confirmation of these results *in vivo* is, unfortunately, not possible as mice do not have the CD58 gene and CD2 expression in mouse and human is different (9).

OX40 signaling facilitates prolonged cytokine synthesis through the stabilization of cytokine mRNA transcripts (Fig. 8). The 3′ UTR of many short-lived transcripts, including those for cytokine genes such as IL-2, IL-3 and IFN-γ, contains AUUUA elements that act as recognition sites for several RNA-binding proteins (32, 33). Activation of these proteins leads to the stabilization or destabilization of the targeted mRNA transcript, a process that is under the control of several signal transduction pathways. Our findings of a role for the PI3K-Akt/PKB and p38 MAPK pathways are completely in agreement with a previous report demonstrating that these two pathways act in parallel to regulate IL-3 mRNA stability in NIH 3T3 cells (34). In this instance, p38 was shown to target the stabilizing protein, HuR; the PI3K target was not identified. Also consistent with our data is the recent identification of PKB (Akt) as an immediate downstream target of OX40 signaling in mouse T cells (23).

Although the IL-10 3′UTR also contains AREs, we saw no effect of OX40 signaling on mRNA stability and no effect of any of the inhibitors (data not shown). This is not without precedent as a recent genome-wide survey of transcripts containing AUUUA elements found that at least 10% of these genes had long-lived transcripts (38). This implies that AREs are not always predictive of rapid mRNA turnover and regulation of transcript stability. The IFN-γ gene appears to have intermediate sensitivity to the PI3K and p38 inhibitors, suggesting further complexity in the regulation of cytokine mRNA stability.

Our finding of OX40/PKB-mediated stabilization of cytokine mRNA suggests that T cell longevity may be regulated by OX40 through more than one mechanism—protection from apoptosis and sustained expression of cytokines that drive proliferation. Indeed, blocking OX40 signaling only partially suppressed T cell proliferation (Fig. 3), suggesting an important role for other cytokines, such as IL-7 and IL-15, that are presumably independent of OX40. Collectively, our results and those of others suggest that early co-stimulatory signals, such as CD2 engagement, act to enhance TCR-mediated signaling and augment cytokine transcription, whereas late co-stimulatory signals, such as OX40 stimulation, serve to prolong ongoing responses, at least in part, by post-transcriptional mechanisms.

The observations that OX40L is found on human vascular ECs at sites of inflammation *in vivo* and that OX40–OX40L interaction directly regulates the expression of T cell cytokines suggest that OX40 may play an important role in the activation of T cells at sites of inflammation. In this study, we show that OX40 stimulation regulates the expression of IL-2, IFN-γ and IL-3, cytokines that are known to mediate T cell growth and promote a state of inflammation. IL-3 is produced by activated T cells and enhances the proliferation of cultured ECs (39). It also induces the expression of E-selectin and P-selectin, resulting in increased adhesion of $CD4^+$ T cells to the ECs (39, 40). It has recently been shown that IFN-γ induces IL-3R on ECs and that the combination of IL-3 and IFN-γ has a synergistic effect on the up-regulation of EC MHC class II expression (41). These findings suggest that IL-3 along with IFN-γ may play an important role in sustaining ECs in an activated state during the process of chronic inflammation. The data presented in this paper provide additional support for the idea that human ECs play an important role in activating resting memory $CD4^+$ T cells at sites of infection in the periphery and that EC–T cell crosstalk through OX40L and cytokines may aid in regulating the persistence of $CD4^+$ T cell responses at the sites of inflammation.

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Abbreviations

ActD	actinomycin D
APC	antigen-presenting cell
ARE	AU-rich elements
EC	endothelial cell
HUVEC	human umbilical vein endothelial cells
ICOSL	inducible costimulator ligand
JNK	c-jun-N-terminal kinase
MAPK	mitogen-activated protein kinase
PI3K	phosphotidyl-inositol-3-kinase
PKB	protein kinase B
SAg	superantigen
TNF	tumor necrosis factor
TSST-1	toxic shock syndrome toxin-1
UTR	untranslated region

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CTLA-4 gene polymorphism is not associated with conventional multiple sclerosis in Japanese

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Abstract

We investigated the polymorphisms of exon 1 (+49A/G) and promoter (−318C/T and −651C/T) regions of the CTLA-4 gene in 133 Japanese patients with conventional/classical multiple sclerosis (MS) and 156 healthy controls. Patients with optico-spinal MS (OSMS) or atypical clinical attacks were excluded from the study. There was no significant difference in the distribution of polymorphisms between patients and controls. Furthermore, there were no associations between polymorphisms and clinical characteristics, such as age at onset, disease prognosis, and HLA profiles. Our results suggest that CTLA-4 gene polymorphisms are neither conclusively related to susceptibility nor to the clinical characteristics of MS, especially in Japanese patients with conventional/classical form and clinical features identical to those of their counterparts in Western countries.

Keywords: CTLA-4 gene polymorphism; HLA-DRB1*1501; Multiple sclerosis; Disease susceptibility; Disease prognosis; Japanese

1. Introduction

Multiple sclerosis (MS) is considered as an autoimmune disease, and susceptibility to this condition is controlled by multiple genes and environmental factors (Vyse and Todd, 1996). Despite evidence for a strong genetic influence, a weak major histocompatibility complex (MHC) association is the only consistently observed genetic feature in MS (Hillert, 1994; Compston et al., 1995), and recent genome wide linkage studies demonstrated that MS follows a polygenetic trait with multiple loci (Ebers et al., 1996). The genes involved in polygenic diseases like MS are not easily identified because clinical manifestation requires several disease-associated alleles of several genes rather than one specific mutation. The analysis of multifactorial diseases like MS is further complicated by the fact that functional differences of known polymorphisms have not yet been identified.

CTLA-4 gene is a strong candidate gene for involvement in autoimmune diseases because it plays an important role in the termination of T cell activation (Waterhouse et al., 1995; Ueda et al., 2003). The CTLA-4 gene is located on chromosome 2q33 region, a region recognized as a candidate locus by linkage genome scan (Ebers et al., 1996). Several polymorphisms in the CTLA-4 locus have been reported, and several studies have addressed the potential role of single nucleotide polymorphism (SNP) in exon 1 (+49A/G), a microsatellite (AT)n marker at position 642 of exon 4, and SNPs in the promoter regions (−318C/T and −651C/T) of the CTLA-4 gene in susceptibility to MS with different results in different ethnic groups (Harbo et al., 1999; Ligers et al., 1999; Andreevskii et al., 2002; Rasmussen et al., 2001; Dyment et al., 2002; Maurer et al., 2002; van Veen Tineke et al., 2003; Kantarci et al.,

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2003). Interactions between CTLA-4 gene and HLA DR2 in the development of MS were also reported (Rasmussen et al., 2001; Alizadeh et al., 2003). In contrast, we previously found no association between exon 1 (+49A/G) SNP and MS in 74 Japanese patients and 93 controls, although the polymorphism was suggested to modulate the disease (Fukazawa et al., 1999). In our previous study, however, only exon 1 (+49A/G) SNP was investigated, and the subjects of the study included patients with clinically or paraclinically atypical attacks, as described previously (Fukazawa et al., 1999; 2004).

The aims of the present study were to analyze the relationships of three CTLA-4 gene polymorphisms [exon 1 (+49A/G) and promoter regions (−318C/T and −651C/T)] with disease onset and disease prognosis in an expanded data set of 133 Japanese patients with MS. We also investigated whether the CTLA-4 gene polymorphism interacts with HLA-DRB1*1501 in the development of MS. Patients with optico-spinal MS (OSMS) were excluded from the present study. Patients with atypical clinical or paraclinical findings (Fukazawa et al., 2004) were also excluded, and thus, the clinical features of the selected patients were identical to those in Western countries. All subjects studied were residents of Hokkaido, the northernmost island of Japan.

2. Patients and methods

2.1. Subjects

The study subjects were 133 unrelated Japanese patients with conventional/classical MS (CMS) who met the inclusion and exclusion criteria described below. All patients exhibited two or more clinical attacks and had objective clinical evidence of multiple lesions without any evidence of other disorders. They also fulfilled the diagnostic criteria for MS (Poser et al., 1983; McDonald et al., 2001). All patients showed a relapsing–remitting or secondary progressive course. Patients with neuromyelitis optica (NMO) or optico-spinal MS (OSMS) were excluded. Patients with clinically or paraclinically atypical attacks were also excluded because they have been reported to be a clinically and immunogenetically distinct subtype among patients with diagnosis of MS (Fukazawa et al., 2003; 2004). The definitions of OSMS and atypical attacks were described previously (Yamasaki et al., 1999; Fukazawa et al., 2000; 2003; 2004). Therefore, in the current study, all patients studied were classified as having "conventional/classical MS (CMS)" with involvement of multiple sites in the CNS, including the cerebrum, cerebellum, or brainstem, with clinical features similar to those observed in Western countries (Fukazawa et al., 2000; 2004; Weinshenker, 2003). Among 133 patients studied, 61 patients had participated in our previous study and were analyzed for exon 1 A/G polymorphisms (+49; Fukazawa et al., 1999). The control group comprised 156 healthy Japanese volunteers. All study participants were Japanese and were resident of Hokkaido, the northernmost island of Japan. Their ancestors were from various parts of Japan, since Hokkaido was first reclaimed around 1870. The native inhabitants of Hokkaido are said to be the Ainu tribe, but this remains a controversial issue partly due to lack of information on the origin of this tribe. Informed consent was obtained from each individual in writing at the time of blood sampling.

2.2. Analysis of CTLA-4 polymorphism and HLA-typing

A blood sample was obtained, and high molecular weight DNA was extracted from peripheral blood cells. Exon 1 A/G polymorphisms (+49) were determined using the method described previously (Fukazawa et al., 1999). Genotypes at polymorphic sites −318 and −651 in the promoter region of the CTLA-4 gene were determined by polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP). The genotype at position −318 in the promoter region was identified as described previously (Rasmussen et al., 2001). The −651 SNP was detected using mismatch primers with sequences 5′-ttttatggacggctctaatctc-3′ and 5′-agaaaaaaatcacaagaaataaactgaaaatagc-3′. The amplified products were digested with *Msp*I (Boehringer Mannheim, Mannheim, Germany) and analyzed on 3% agarose gel. The C allele corresponds to the presence of two 42- and 144-bp fragments generated by *Msp*I digestion, and the T allele corresponds to the 186-bp uncleaved fragment with no *Msp*I site. Exon 1 (+49) and the promoter (318) dimorphisms were determined by using the protocol described previously (Harbo et al., 1999). We used primers with sequences 5′-TCTTTTCCGCCTATTTTCAGTT-3′ and 5′-CCCTGGAATACAGAGCCAGC-3′, and the amplified products were treated with the restriction enzymes *Tse*I and *Mse*I. On agarose gel electrophoresis, the haplotype combination of the polymorphic positions at +49 and 318 was identified by the 626-bp fragment (corresponding to the 318C, +49 A haplotype), 530-bp fragment (corresponding to the 318 T, +49 A haplotype), 460-bp fragment (corresponding to the 318 C, +49 G haplotype), and 365-bp fragment (corresponding to the 318 T, +49 G haplotype).

DNA typing of DRB1 alleles was analyzed by the nonisotopic oligotyping method using reverse dot blot hybridization. When the discrimination was not clear by the reverse dot blot hybridization method, we used the standard PCR-specific oligonucleotide probe (PCR-SSOP) method.

2.3. Disease prognosis

MS severity was defined according to the expanded disability status scale of Kurtzke (EDSS), progression index (PI), and ranked severity score (RSS). PI was calculated as a

measure of accumulated disability over time (PI=EDSS/disease duration in years), and then we determined RSS for patients with disease duration longer than 5 years (Rodriguez et al., 1994; Weinshenker et al., 1997). The RSS describes disability, and the score is inversely related to the EDSS. Since disease progression in the early stages of MS may be variable and not reflect the potential for prognosis in the longer term, we analyzed CTLA-4 gene-polymorphism associations with severity in patients with disease duration longer than 5 years.

2.4. Statistical analysis

Allele frequencies and genotype frequencies of the CTLA-4 gene were compared between MS patients and controls, using the chi-square test or Fisher's Exact Test. Phenotype frequencies of HLA-DRB1*1501 were also examined for association with conventional MS. To investigate the potential interactions between the DRB1*1501 allele and the CTLA-4 alleles, allele frequencies and genotype frequencies of the CTLA-4 gene were compared separately among DRB1*1501-positive and -negative subjects. The p value was multiplied by 2 to correct for stratification according to DRB1*1501 status. We analyzed CTLA-4 gene-polymorphism associations with PI and RSS using Kruskal–Wallis test. Statistical analysis was performed with StatView version 5.0 (Abacus Concept, Berkeley, CA).

3. Results

3.1. Clinical profile of patients studied

Patients consisted of 43 men and 90 women (male/female ratio=0.48). The mean age at blood sampling was 35.0 years (S.D.=10.7; range: 14–67). The mean age at onset was 27.1 years (S.D.=9.6; range: 4–57). The mean duration of disease was 11.5 years (S.D.=8.7; range: 1–37). The EDSS ranged from 0.0 to 9.5 (mean=3.0; S.D.=2.6). Clinical features of these cases with conventional MS were quite similar to those of MS in Western populations (Weinshenker, 2003; Fukazawa et al., 2004). The control subjects were Japanese of ethnic background similar to that of the study group and consisted of 52 men and 104 women (male/female ratio=0.50), and their mean age at blood sampling was 33.5 years (S.D.=9.2; range: 20–58). The sex ratio and the mean age of patients were not significantly different from those of the control.

3.2. CTLA-4 gene polymorphisms

The genotype and allele frequencies of CTLA-4 gene exon 1 +49 SNP, promoter −318 SNP, and the haplotypes of the 2 alleles are shown in Tables 1 and 2. In control subjects, the genotype frequencies conformed to Hardy–Weinberg expectations. The distributions of CTLA-4 exon 1 +49 and promoter −318 genotypes and allele frequencies were similar in CMS patients and controls. The frequencies of the CTLA-4 haplotypes studied were also similar in patients and controls (Table 1). Only the T allele was found at promoter −651 position in our Japanese population without polymorphism.

3.3. HLA-DRB1*1501 positivity and its interaction with CTLA-4 gene

A significantly higher frequency of DRB1*1501 was found in MS patients (p=0.0033; odds ratio=2.29; 95%CI=1.32–3.98) than in controls. Among HLA-DRB1*1501-positive subjects, the CTLA4 exon 1 +49 genotypes and haplotypes were almost equally distributed between patients and controls (Table 1). The frequency of C/T genotype at promoter −318 was higher in DRB1*1501-positve patients (16/43; 37.2%) than in DRB1*1501-positive controls (4/27; 14.8%), but the difference was not significant (corrected p=0.139; Table 1). Allele frequencies of CTLA-4 exon 1 +49 and promoter −318 SNP were also equally distributed between patients and control subjects among HLA-DRB1*1501-positive subjects (Table 2).

3.4. Clinical characteristics and CTLA-4 polymorphism

Among the 133 conventional MS patients, there were no associations between exon1 +49, promoter −318 polymorphisms, and haplotypes and clinical characteristics. The clinical characteristics were age at disease onset and clinical course (relapsing and remitting or secondary progressive; data not shown). Disease prognosis was analyzed for 97

Table 1
Distribution of CTLA-4 exon 1 +49 and promoter −318 genotypes and haplotypes in conventional MS patients and controls

	Patients		Control	
	Total	DRB1*1501 +	Total	DRB1*1501+
	n=133	n=43	n=156	n=27
Exon 1 +49 genotypes				
AA	23 (17.3)	8 (18.6)	29 (18.6)	5 (18.5)
AG	69 (51.9)	23 (53.5)	66 (42.3)	14 (51.9)
GG	41 (30.8)	12 (27.9)	61 (39.1)	8 (29.6)
Promoter −318 genotypes				
CC	100 (75.2)	27 (62.8)	124 (79.5)	22 (81.5)
CT	32 (24.1)	16 (37.2)	28 (17.9)	4 (14.8)
TT	1 (0.8)	0 (0.0)	4 (2.6)	1 (3.7)
Haplotypes				
CA	81 (30.5)	23 (26.7)	88 (28.2)	18 (33.3)
CG	151 (56.8)	47 (54.7)	188 (60.3)	30 (55.6)
TA	34 (12.8)	16 (18.6)	36 (11.5)	6 (11.1)
TG	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

Numbers in parentheses represent percentage of subjects.
No significant differences were detected between patients and control.

Table 2
Allele frequencies of CTLA-4 exon 1 +49 and promoter −318 SNP in patients with conventional MS and control subjects by DRB1*1501 status

	Patients		Control	
	Total	DRB1*1501+	Total	DRB1*1501+
	n=266	*n*=86	*n*=312	*n*=54
Exon 1 +49 genotypes				
A allele	115 (43.2%)	39 (45.3%)	124 (39.7%)	24 (44.4%)
G allele	151 (56.8%)	47 (54.7%)	188 (60.3%)	30 (55.6%)
Promoter −318 genotypes				
C allele	232 (87.2%)	70 (81.4%)	276 (88.5%)	48 (88.9%)
T allele	34 (12.8%)	16 (18.6%)	36 (11.5%)	6 (11.1%)

SNP—single nucleotide polymorphism.
No significant differences were detected in allele frequencies between the two groups.

patients, and CTLA-4 polymorphisms were not associated with prognosis indicated by PI or RSS (data not shown).

4. Discussion

We found no differences in CTLA-4 polymorphisms between patients and controls irrespective of DRB1*1501 status. Furthermore, our present results indicated that CTLA-4 polymorphisms are not associated with the disease prognosis.

Several studies have examined the association between CTLA-4 gene polymorphisms and MS. There was no evidence for CTLA-4 as a susceptibility gene in Caucasians from Denmark (Rasmussen et al., 2001), Canada (Dyment et al., 2002), Germany (Maurer et al., 2002), Netherlands (van Veen Tineke et al., 2003), and Russia (Andreevskii et al., 2002). On the other hand, exon 1 +49AG genotype was associated with MS in Norwegian (Harbo et al., 1999), and GG genotype in Swedish populations (Ligers et al., 1999). Furthermore, an American study showed the association of MS with the homozygous common haplotype A (+49)/C (−318)/AT_8 (−642) (Kantarci et al., 2003). Furthermore, the interactions of HLA-DR2 with A (+49)/C (−318) haplotype and C/T SNP in the promoter region (−651) of the CTLA-4 gene in the development of MS were reported in Chinese (Rasmussen et al., 2001) and European Caucasians (Alizadeh et al., 2003), respectively. In addition, polymorphisms have been suggested to influence the clinical course or disability (Harbo et al., 1999; Maurer et al., 2002). Therefore, the role of CTLA-4 gene polymorphisms seems to differ among various ethnic groups. On the other hand, our previous study on Japanese population showed no association between MS and CTLA-4 gene exon 1 A/G +49 polymorphism but suggested that the polymorphism may modulate the disease (Fukazawa et al., 1999). The present study again failed to detect associations between CMS and CTLA-4 polymorphisms irrespective of HLA-DRB1*1501 status. Accordingly, we conclude that CTLA-4 gene does not correlate with risk of CMS in Japanese. The present study could not confirm that CTLA-4 gene polymorphisms modulate the disease process. In our previous study, the number of subjects studied was too small to investigate patient prognosis. Furthermore, our previous study included patients with clinically or radiologically atypical attacks, and those atypical patients were recently suggested to constitute a distinct subgroup among Japanese patients with a diagnosis of MS (Fukazawa et al., 2003; 2004). Therefore, CTLA-4 gene is less likely to influence the prognosis of patients, especially those with conventional/classical form of MS, whose clinical features are identical to those of their Western counterparts. In contrast, previous studies implicated CTLA-4 gene in susceptibility to diabetes mellitus (Marron et al., 1997; Van der Auwera et al., 1997), and one of these studies indicated that the transmission of SNPs in regulatory regions was crucial in this process (Marron et al., 1997). Accordingly, exclusion of the role of CTLA-4 may require a dense SNP association study. Furthermore, since MS is clinically, pathologically, and immunogenetically heterogeneous, the possible relationships between clinical subtypes and the role of CTLA-4 polymorphisms should be further investigated in larger populations.

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Short communication

Platelet-activating factor receptor gene polymorphism in Japanese patients with multiple sclerosis

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Abstract

We evaluated the association of the platelet-activating factor receptor (PAFR) gene polymorphism (A224D) with the susceptibility and severity of multiple sclerosis (MS) in a Japanese population. DNA was collected from 162 Japanese patients with clinically definite 'conventional' MS (MS) and 245 healthy controls. The missense mutation A224D that impairs PAF-PAFR signaling was determined by polymerase chain reaction restriction fragment length polymorphism. The frequency of the AD/DD genotypes was significantly higher in MS patients (21.0%) than in healthy controls (13.5%) (p=0.045; odds ratio (OR), 1.71; 95% confidence interval (CI), 1.01–2.89). Moreover, the frequency of D allele in MS patients (11.7%) was also significantly higher than those in healthy controls (6.9%) (p=0.019; OR, 1.78; 95% CI, 1.10–2.89). These findings suggest that the PAFR gene missense mutation has a relation to the susceptibility for MS.

Keywords: Polymorphism; Platelet-activating factor receptor; Multiple sclerosis; Japanese; Missense mutation

1. Introduction

Increased levels of platelet-activating factor (PAF) receptor (PAFR) mRNA are reported in multiple sclerosis (MS) plaques together with several genes encoding molecules associated with allergic responses (Lock et al., 2002). In experimental allergic encephalomyelitis (EAE), an animal model of MS, transcripts for PAFR were reported as elevated in the CNS during the disease, and PAFR antagonist was shown to reduce the severity of EAE (Pedotti et al., 2003). Moreover, in cerebrospinal fluid and plasma of patients with the relapsing–remitting MS, elevation of PAF that correlated with the number of gadolinium-enhancing lesions was found on brain MRI (Callea et al., 1999). Taken together, the above findings are suggestive that PAF might have a proinflammatory role in MS.

PAF is a very potent chemotactic stimulant for inflammatory cells such as eosinophils (Wardlaw et al., 1986) and polymorphonuclear neutrophils (O'Flaherty et al., 1981). PAF not only promotes leukocyte adhesion and transmigration by the induction of intracellular adhesion molecule-1 (ICAM-1) on endothelial cells (Chihara et al., 1992), but also upregulates major histocompatibility complex (MHC) class I and II expressions in some brain cells that are critical in antigen presentation (Martin-Mondière et al., 1987). These proinflammatory and vasoactive actions of

Abbreviations: PAFR, platelet-activating factor receptor; MS, multiple sclerosis; OR, odds ratio; CI, confidence interval; EAE, experimental allergic encephalomyelitis; ICAM-1, intracellular adhesion molecule-1; MHC, major histocompatibility complex; EDSS, Kurtzke's Expanded Disability Status Scale; PI, progression index; TNF-α, tumor necrosis factor-alpha; IL, interleukin; TGF-β, transforming growth factor-beta; DTH, delayed-type hypersensitivity.

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