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.
OX40 signaling stabilizes T cell cytokine mRNA

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). Protein secretion was also measured 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.

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). CD4+ T cells were plated on a monolayer of class II+ ECs, pre-treated 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 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.

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 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). 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 t1/2 values were calculated at either 2 or 3 h post-ActD and were derived according to the
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.

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

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 plates. 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 OX40L (Alexis Biochemicals). Where indicated, cells were cultured, as above, in the presence of either 10^5 T cells and ECs, respectively. OX40 expression was low or absent on resting 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.
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.
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 (293OX40L) and a control cell line (293vector) 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 293OX40L cells in providing co-stimulation, we stimulated CD4+ T cells with plate-bound anti-CD3 mAb in the presence of either 293OX40L or 293vector cells and analyzed supernatants for IL-2 secretion after 24 h. In the presence of 293vector 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 293OX40L cells, IL-2 secretion was augmented ~3-fold and disruption of OX40–OX40L interaction completely blocked this effect. We next examined the ability of 293OX40L 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 293OX40L cells or ECs, respectively (Fig. 5C). Again, 293OX40L cells provided a strong co-stimulatory signal and the combination of 293OX40L 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 293OX40L cells provide a useful tool to isolate the co-stimulatory actions of OX40L from those of other molecules found on ECs such as ICOSL.

OX40 signaling augments T cell cytokine mRNA levels

Using the 293OX40L 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 293OX40L cells rather than ECs as this ensured minimal interference from other co-stimulatory molecules.
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 293OX40L or 293vector 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 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 (C255 IL-2-luc) and stimulated them with PHA in the presence of either 293OX40L or 293vector 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 (C255 2100IL-2-luc). This reporter was also unresponsive to OX40 signals (Fig. 7B), although interestingly, luciferase activity did not decrease over
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 293OX40L or 293vector 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 293OX40L or 293vector cells

![Graphs showing mRNA stabilization](https://example.com/graphs.png)
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

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

<table>
<thead>
<tr>
<th></th>
<th>293vector</th>
<th>293OX40L</th>
<th>293OX40L + Inh.</th>
<th>% Decrease</th>
<th>Number of experiments</th>
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<tr>
<td>p38 Inh.</td>
<td>0.98</td>
<td>4.18</td>
<td>1.37</td>
<td>88 ± 7.5</td>
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<tr>
<td></td>
<td>0.75</td>
<td>2.41</td>
<td>0.83</td>
<td>85 ± 10.5</td>
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</tr>
<tr>
<td>IFN-γ</td>
<td>0.74</td>
<td>2.19</td>
<td>2.02</td>
<td>16 ± 18</td>
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aShown 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 293vector or 293OX40L 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.

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 293vector or 293OX40L. 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.
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.

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.
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, 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 alone 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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