

## Expression of gp34 (OX40 Ligand) and OX40 on Human T Cell Clones

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**gp34, which we previously cloned, is a ligand of OX40 (CD134), a costimulatory molecule involved in T cell activation. To elucidate the role of human OX40/OX40L interaction, we examined the expression of gp34 (OX40L) and OX40 in normal human hematopoietic cells by using flow cytometry. OX40 expression is observed on activated T cells, while OX40L is expressed in antigen-presenting cells. However, cytotoxic T lymphocyte (CTL) clones specific for Epstein-Barr virus (EBV)-transformed autologous lymphoblastic cell lines (LCLs) induced both OX40 and OX40L expression after antigen or T cell receptor (TCR) stimulation. This study suggests a possible function of OX40L/OX40, through T cell-T cell interaction, in the reactivation of memory T cells in an auto-crine manner, with implications for the pathogenesis of viral infections and neoplasms.**

Key words: gp34 — OX40 ligand (OX40L) — OX40 — T cell clones

OX40 (CD134), a member of the tumor necrosis factor (TNF) receptor superfamily, is preferentially expressed on normal activated T cells in human, rat and mouse.<sup>1–7</sup> Co-stimulatory signal transduction of OX40 has been demonstrated to be involved in proliferation and cytokine production of activated T cells *in vitro*.<sup>1–3, 6, 7</sup> Further investigations have revealed the presence of OX40-expressing T cells at inflammatory sites of various diseases, such as neoplasms,<sup>8</sup> graft-versus-host-disease (GVHD),<sup>9–11</sup> and experimental allergic encephalomyelitis (EAE).<sup>12</sup> OX40 ligand (OX40L), a type II membrane protein, was identified as the mouse homologue of human gp34 that we molecularly cloned as a target molecule for a trans-acting transcriptional activator, Tax, of the human T cell lymphotropic virus type I (HTLV-I).<sup>6, 13–15</sup> Expression of OX40L, initially described on HTLV-I-infected T cell lines,<sup>13, 14</sup> has been found on murine B cells,<sup>16–19</sup> human endothelial cell lines,<sup>20</sup> dendritic cells (DCs),<sup>19, 21, 22</sup> and among a population of antigen-presenting cells (APC) in the central nervous system during the course of clinically apparent EAE.<sup>23</sup>

Recently, we and others have demonstrated impairment of antigen-specific activation of T cells in both OX40L- and OX40-deficient mice due to dysfunction of APC and T cells, respectively.<sup>19, 24, 25</sup> These results suggest that the

interaction between OX40L expressed on professional APC and OX40 expressed on activated T cells is crucial for antigen-specific T cell activation through APC function *in vivo* and *in vitro*. A number of reports have demonstrated the functional importance of OX40L in activating T cells. However, little is known about the functional significance of OX40L expression on T cells including HTLV-I-infected T cells, especially since either freshly isolated T cells or stimulated T cells seemed to express little, if any, OX40L. In this context, our present study, demonstrating that antigen stimulation induces expression of OX40L on human cytotoxic T lymphocyte (CTL) clones, is an important finding with clear implications for the function of the OX40L/OX40 system in T cell-T cell interactions in immune responses, particularly with regard to the control of viral infections and tumors.

We first analyzed the expression of OX40L and OX40 on normal human PBMCs. No appreciable expression of OX40L was seen on CD3<sup>+</sup> T cell and CD19<sup>+</sup> B cell populations of unstimulated PBMCs (Figs. 1 and 2A). Stimulation with anti-CD3 monoclonal antibody (mAb) (Fig. 1), phytohemagglutinin (PHA), phorbol ester (PMA)/ionomycin, or anti-CD3 plus anti-CD28 (data not shown) was not effective in inducing expression of OX40L on CD4<sup>+</sup> or CD8<sup>+</sup> T cell populations, as shown previously,<sup>14</sup> while CD19<sup>+</sup> B cell populations clearly expressed OX40L after stimulation with anti-CD40 mAb and anti-IgM polyclonal Ab (Fig. 2A) or pokeweed mitogen (PWM) (data not shown). OX40 expression was seen in a portion of CD4<sup>+</sup> T cells, but not CD8<sup>+</sup> T cells or B cells before treat-

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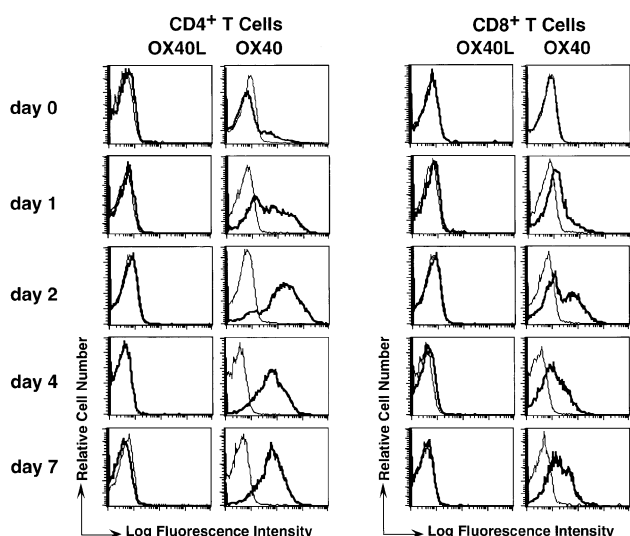


Fig. 1. Induction of OX40L and OX40 on T cells among anti-CD3-stimulated T cells. Human PBMCs were treated with immobilized anti-CD3 mAb (PharMingen, San Diego, CA) for the indicated days. The cells were double-stained with FITC-anti-CD4 or FITC-anti-CD8 in combination with either biotinylated anti-OX40L mAb (TAG34) or biotinylated anti-OX40 mAb (Ber-ACT35) followed by streptavidin-APC. CD4<sup>+</sup> and CD8<sup>+</sup> T cells were separately gated and APC staining was analyzed with FACScan. Thick lines indicate results of staining with biotinylated TAG34 or Ber-ACT35 and thin lines indicate biotinylated mouse IgG1 (control) staining. The results are representative of five independent experiments using PBMCs from ten healthy adults.

ment with mitogens. Profound expression of OX40 was induced by stimulation of PBMCs with anti-CD3 mAb on CD4<sup>+</sup> T and CD8<sup>+</sup> T cells, reaching a maximum on day 2 of stimulation, with a gradual decrease thereafter (Fig. 1). Stimulation of PBMCs with PHA or PMA/ionomycin also resulted in induction of OX40 expression on CD4<sup>+</sup> T and CD8<sup>+</sup> T cells (data not shown). Addition of anti-CD28 mAb to anti-CD3 stimulation did not increase the expression level and kinetics of OX40 on T cells as compared with anti-CD-3 alone in spite of a previous report showing that CD28 signaling enhanced the expression level of OX40 after stimulation with anti-CD3 in mice.<sup>26)</sup> Stimulation of PBMCs with anti-CD40 mAb and anti-IgM polyclonal Ab resulted in no induction of OX40 expression on CD19<sup>+</sup> B cells (Fig. 2A), though stimulation with PWM induced significant OX40 expression on CD19<sup>+</sup> B cells (data not shown).

We next investigated the expression of OX40L on human natural killer (NK) cells, and peripheral monocyte-derived DCs. Upon stimulation with interleukin (IL)-2, the CD16<sup>+</sup>CD56<sup>+</sup> NK cell population showed no OX40L expression, but developed significant OX40 expression

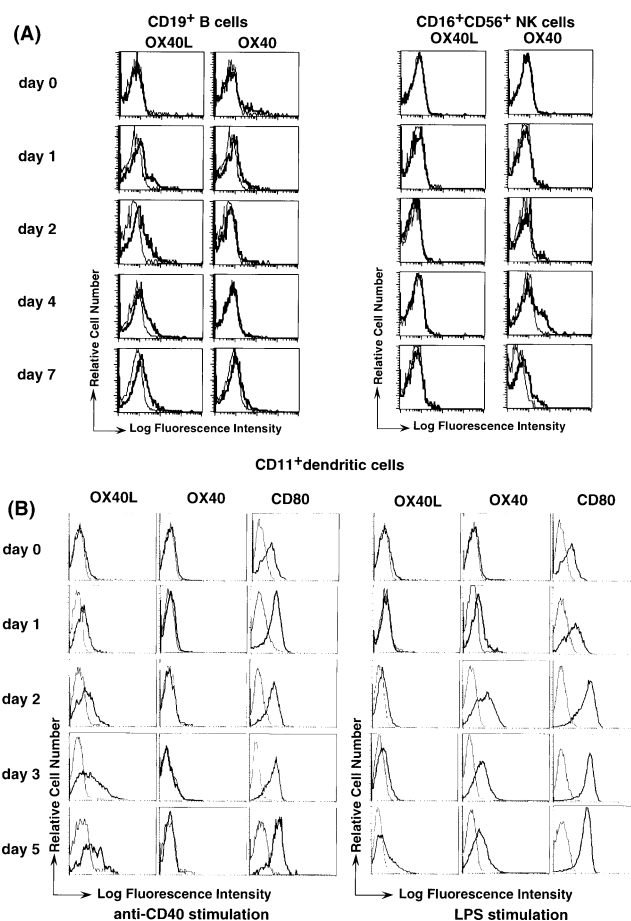


Fig. 2. A. Induction of OX40L and OX40 on activated B and NK cells. For B and NK cell activation, human PBMCs were treated with anti-CD40 mAb (Mabtech AB, Nacka, Sweden) plus anti-IgM polyclonal Ab (Organon Technika, West Chester, PA) or rIL-2 for the indicated days. The cells were stained with FITC-CD19 (B cells) or FITC-CD16 plus PE-CD56 (NK cells) in combination with either biotinylated anti-OX40L mAb (TAG34) or biotinylated anti-OX40 mAb (Ber-ACT35) followed by streptavidin-APC. CD19<sup>+</sup> B or CD16<sup>+</sup>CD56<sup>+</sup> NK cells were separately gated and APC staining was analyzed with FACScan. Thick lines indicate results of staining with biotinylated TAG34 or Ber-ACT35 and thin lines indicate biotinylated mouse IgG1 (control) staining. The results are representative of five independent experiments using PBMCs from ten healthy adults. B. Induction of OX40L and OX40 on activated DCs. To isolate monocyte-derived DCs, the adherent cells from human PBMCs were treated with GM-CSF and IL-4 for 5 days. The detached cells were incubated in the presence of anti-CD40 mAb (Mabtech AB) or LPS for the indicated days. The cells were double-stained with PE-CD11c in combination with FITC-CD80, biotinylated anti-OX40L mAb (TAG34) or biotinylated anti-OX40 mAb (Ber-ACT35) followed by streptavidin-APC. CD11c<sup>+</sup> activated dendritic cells were separately gated and analyzed as described in the legend to Fig. 1. Thick lines indicate results of staining with biotinylated TAG34, Ber-ACT35 or FITC-CD80 and thin lines indicate biotinylated or FITC-conjugated mouse IgG1 (control) staining. The results are representative of four independent experiments using PBMCs from four healthy adults.

after 4 days of stimulation (Fig. 2A). Anti-CD40 mAb stimulated DCs significantly upregulated the expression of OX40L, but not OX40. On the other hand, lipopolysaccharide (LPS) stimulation clearly induced the DC to express both OX40L and OX40 (Fig. 2B). These results are the first evidence showing OX40 is expressed by non-T cells. Both anti-CD40 mAb and LPS were equally able to induce CD80 expression on the activated DC (Fig. 2B). This result suggests that OX40 signaling might affect APC function as well as OX40L expression. To elucidate the functional role of OX40 on APCs, further study will be necessary.

To examine the expression of OX40 and OX40L at the single cell level in long-term-cultured T cells, we established human CTL clones specific for Epstein-Barr virus (EBV)-transformed autologous LCL cells. EBV-transformed lymphoblastic cell lines (LCLs) and human CTL clones specific for the autologous LCL were prepared as described previously.<sup>15,27</sup> Briefly, human PBMCs were stimulated with the irradiated autologous LCL cells for 7 days. OX40 was expressed on activated T cells at this stage while no OX40L was seen (data not shown). Three further rounds of restimulation in the presence of autologous LCL cells were conducted, and three CD4<sup>+</sup> T cell clones, CT4-1, CT4-2 and CT4-3, and a CD8<sup>+</sup> T cell clone, CT8-1, were established by limiting dilution. These

clones were cultured for 14 days in the presence of autologous LCL cells and IL-2. All of the CTL clones, which showed little or no proliferation in response to IL-2 (data not shown), indicating a resting state, were examined for expression of OX40L and OX40. No expression of OX40L or OX40 was detectable at this stage (Fig. 3). Surprisingly, OX40L expression was clearly induced on all the CTL clones a day after restimulation and peaked transiently on day 2 (Fig. 3). CT4-2 and CT4-3 showed lower induction of OX40L expression as compared with other clones. Expression of OX40, however, varied among the CTL clones; significant induction of OX40 was seen in CT4-1 and CT4-2 on day 2 after stimulation, whereas CT4-3 and CT8-1 showed distinct but low levels of expression of OX40 during stimulation for 7 days (Fig. 3). Expression levels of CD25 on each clone were similar during antigen stimulation (data not shown), suggesting that all the clones were activated normally in spite of the different expression levels of OX40L and OX40.

One possibility is that LCL stimulator cells produce certain cytokines which are able to induce OX40L expression. To exclude this possibility, two of these CTL clones, CT4-2, CT4-3 were stimulated with anti-CD3 mAb instead of LCL stimulator cells. Expression of OX40L was induced on both CT4-2 and CT4-3 clones after the stimulation with anti-CD3 mAb, with similar kinetics to that

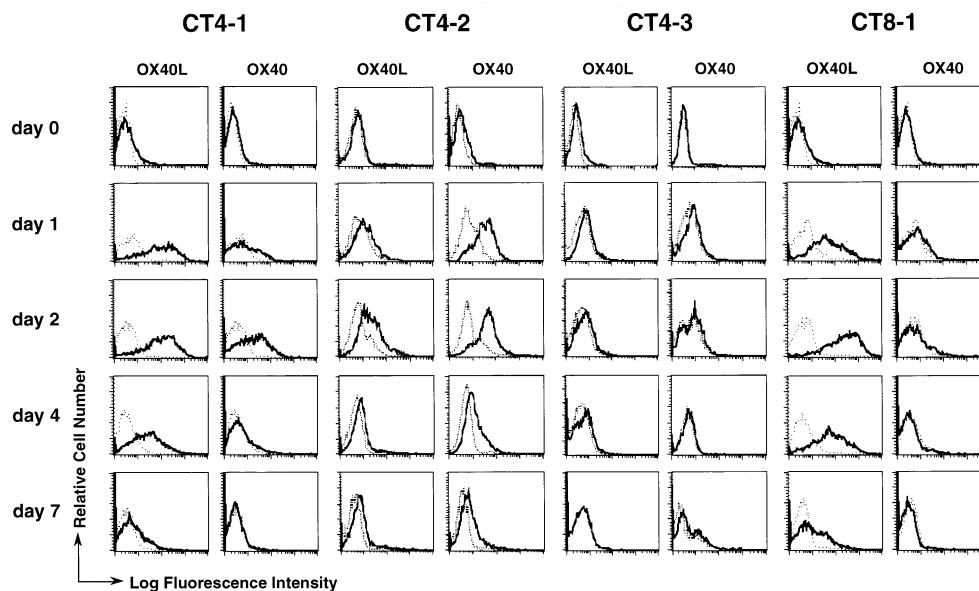


Fig. 3. Induction of OX40L and OX40 on CTL clones upon stimulation with their target cells. Four human CTL clones, CT4-1, CT4-2, CT4-3 and CT8-1, were cultured for the indicated days in the presence of irradiated autologous LCL cells, which are target cells for the CTL clones. The CTL clones were then double-stained with FITC-anti-CD3 mAb in combination with either biotinylated anti-OX40L mAb (TAG34) or biotinylated anti-OX40 mAb (Ber-ACT35) followed by streptavidin-PE. CD3<sup>+</sup> T cells were gated and PE staining was analyzed with FACScan. Solid lines indicate results of staining with biotinylated TAG34 or Ber-ACT35 and dotted lines indicate biotinylated mouse IgG1 (control) staining.

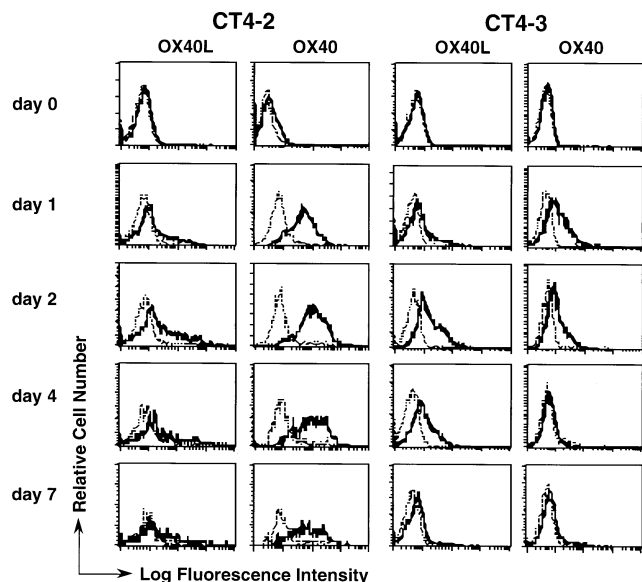


Fig. 4. Induction of OX40L and OX40 on CTL clones upon stimulation with anti-CD3 mAb. Two human CTL clones, CT4-2 and CT4-3 were cultured for the indicated days in the presence of immobilized anti-CD3 mAb. The CTL clones were then double-stained with FITC-anti-CD3 mAb in combination with either biotinylated anti-OX40L mAb (TAG34) or biotinylated anti-OX40 mAb (Ber-ACT35) followed by streptavidin-PE. CD3<sup>+</sup> T cells were gated and PE staining was analyzed with FACSscan. Solid lines indicate results of staining with biotinylated TAG34 or Ber-ACT35 and dotted lines indicate biotinylated mouse IgG1 (control) staining.

seen with LCL stimulator cells (Fig. 4), indicating that stimulation through the T-cell receptors of the CTL clones directly contributes to induction of the expression of OX40L.

We have examined DNA synthesis and IL-2 production of all the CTL clones after antigen stimulation. However, we have not observed a correlation between DNA synthesis or IL-2 production, and expression level of OX40L on each clone (data not shown). Furthermore, we have estimated the cytotoxic activity of the four CTL clones to B-LCL. The three clones, CT4-1, CT4-2, and CT4-3, which showed various levels of OX40L expression, demonstrated no significant difference in CTL activity to B-LCL. Only the CTL activity of the CT8-1 clone, which had high expression of OX40L, was clearly lower than those of the other CD4<sup>+</sup> CTL clones (data not shown). We are unable to conclude, at present, that the OX40L/OX40 interaction plays an important role in CTL induction. To elucidate the effect of OX40L expression on CTL function, further study will be required.

The present study has provided the first evidence that not only OX40, but also OX40L is transiently expressed on normal activated T cells after T cell receptor (TCR)

stimulation. Although soluble OX40-Fc fusion proteins were previously shown to bind weakly to mouse activated T cells, which suggested possible expression of OX40L on the activated T cells,<sup>6</sup> a recent report using a mouse OX40L-specific mAb revealed that OX40L is not expressed on mouse activated T cells.<sup>18</sup> We have confirmed the latter observation using MGP34, a mAb specific for mouse OX40L (data not shown). Similarly, we demonstrated that normal human T cells activated by various T cell stimuli such as anti-CD3, PHA, anti-CD3 plus anti-CD28, and phorbol ester plus ionomycin were unable to induce OX40L expression even after seven days of *in vitro* culture. However, when normal human activated T cells specific for EBV were maintained *in vitro* for a long period of time, they acquired the ability to express both OX40L and OX40 upon TCR stimulation. This suggests that there may be a critical difference between primary and long-term activated T cells in the regulatory mechanism of OX40L expression. We previously demonstrated that OX40L expression is induced by stimulation with anti-CD40 mAb or with anti-CD40 mAb plus anti-IgM Ab in mouse splenic DCs and B cells, respectively.<sup>19</sup> CD40 is known to transduce intracellular signals through activation of NFκB.<sup>28</sup> We have also revealed that HTLV-I Tax induces expression of human OX40L, in which two NFκB-like elements in the OX40L promoter region are involved.<sup>29</sup> These data suggest that NFκB or NFκB-like transcription factors may be involved in the regulation of OX40L expression. However, it is still unknown whether or not such transcription factors are involved in OX40L expression on long-term activated T cells.

OX40L expression was revealed to be inducible upon antigen stimulation in long-term activated T cells, but not naive or primary activated T cells. In other words, memory T cells including CTL clones may express OX40L induced by rechallenge with their specific antigens. This notion is supported by our recent observations demonstrating that the function of memory T cells in OX40L-deficient mice is impaired, although naive T cells show normal reactivity to several mitogens or cytokines.<sup>19</sup> Furthermore, it has also been reported that OX40 not only mediates growth and anti-apoptotic signals in activated human and mouse T cells,<sup>1-4, 6, 7, 30</sup> but OX40L expressed on APC such as activated B and dendritic cells acts as a co-stimulatory molecule to activate human and mouse T cells.<sup>17, 18, 20, 22, 31</sup> We hypothesize that OX40L and OX40 expressed on T cells may play a critical role during both the recall activation of memory T cells through T cell-T cell and T cell-APC interactions. To explain fully the functional significance of OX40L on T cells, further examination will be necessary.

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