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Expression and Function of OX40 Ligand on Human Dendritic Cells[1]

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OX40 ligand (OX40L), a member of the TNF family, was shown to be capable of signaling both the cells on which it is expressed and those expressing OX40, its cognate receptor. Here we show that OX40L is expressed on dendritic cells (DC), the most efficient APC to prime naive T cells. The expression and the functional activity of OX40L were examined by means of mAbs used to stain or cross-link OX40L on 1) freshly isolated human blood DC (bDC) and 2) monocyte-derived DC at different stages of differentiation. These were derived from monocytes cultured either with IL-4 and granulocyte-macrophage CSF (IL-4-Mo-DC) or with IL-4 and granulocyte-macrophage CSF plus TNF-α. Both types of Mo-DC expressed OX40L after stimulation through CD40; ligation of OX40L on activated IL-4-Mo-DC enhanced by 4- to 35-fold their cytokine production (TNF-α, IL-12 p40, IL-1β, and IL-6) and increased CD80, CD86, CD54, and CD40 expression. Stimulation of activated IL-4-Mo-DC through OX40L strikingly enhanced their maturation as evidenced by CD83 up-regulation, CD115 (CSF-1R) down-regulation, and typical morphologic changes. OX40L was constitutively expressed on a subset of bDC, and its ligation slightly enhanced CD40L-stimulated IL-12 production. OX40L was down-regulated after overnight culture and spontaneously reexpressed on a subset of mature bDC ($CD83^{high}$, $CD33^{high}$, $CD11c^{high}$, $CD5^{+}$). Thus, the expression of OX40L on DC suggests a physiologic role of this molecule during T cell priming by virtue of its ability to costimulate both T cell and DC activation and differentiation. ***The Journal of Immunology,*** **1997, 159: 3838–3848.**

T cell-dependent immunity is critically regulated by TNF ligand and TNFR[4] superfamilies. Each member of the two families of receptor-ligand molecules is expressed on activated T cells. They control the interaction of T cells with other T cells, B cells, monocytes, dendritic cells (DC), as well as with nonlymphoid cells such as endothelial cells. The receptor superfamily contains 10 different type I membrane molecules, including the p75 nerve growth factor receptor, p60 TNFR-I, p80 TNFR-II, TNFR-RP/TNFR-III, CD27, CD30, CD40, 4–1BB, Fas/Apo-1, and OX40. With the exception of lymphotoxin-α (a secreted protein), all members of the TNF family of ligands are type II membrane proteins, including TNF-α, lymphotoxin-β, CD27L, CD30L, CD40L, 4–1BBL, FasL, and OX40L (1–5). Interestingly, signals can be transduced not only through the receptors but also through at least some ligands. For example, engagement of CD27L or CD40L was shown to costimulate activated peripheral blood T cell proliferation and cytokine production (6–8).

Recent studies in the mouse have established that OX40/OX40L interaction plays an essential role in T cell-dependent Ab production. In vitro, B cells activated through their Ig receptor or through CD40, express OX40L and become stimulated to proliferate and secrete high levels of Ig after cross-linking of OX40L by its cognate receptor OX40. Signaling through OX40L does not affect isotype switching but, rather, enhances the activity of the Ig heavy chain gene 3′α enhancer (9). In vivo, blocking OX40/OX40L interaction by means of anti-OX40 Abs strongly inhibits primary and secondary IgG, but not the IgM Ab response to T cell-dependent Ag. Treatment with anti-OX40 Abs does not affect the generation of memory B cells or the development of germinal centers, but suppresses the formation of B cell foci in T cell area of lymphoid organs, where activated $OX40^{+}$ T cells accumulate within the first few days after immunization (10). Taken collectively, these in vitro and in vivo studies indicate that signaling through OX40L is of crucial importance for the terminal differentiation of activated B cells into plasma cells producing high levels of Ig. OX40 is mainly expressed on activated T cells; it is induced within the first 24 h after TCR ligation and remains detectable for up to 3 to 4 days (11, 12). In addition, OX40 cross-linking costimulates the proliferation and cytokine production of activated T cells (13). Thus, the OX40/OX40L pair of complementary molecules is a two-way signaling system, with OX40 on activated T cells providing a differentiation signal to B cells, and OX40L on B cells having the potential to costimulate T cells.

Although the cDNAs encoding human OX40 and its ligand have been cloned, relatively little is known about their function and

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[4] Abbreviations used in this paper: TNFR, tumor necrosis factor receptor; DC, dendritic cells; CD27L, CD27 ligand; FasL, Fas ligand; OX40L, OX40 ligand; GM-CSF, granulocyte-macrophage colony-stimulating factor; sCD40L, soluble CD40 ligand; bDC, blood dendritic cells; Mo-DC, monocyte-derived dendritic cells; IL-4-Mo-DC, monocytes cultured with interleukin-4 and granulocyte-macrophage colony-stimulating factor; TNF-Mo-DC, monocytes cultured with tumor necrosis factor and granulocyte-macrophage colony-stimulating factor; SSC, side scatter.

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cellular distribution (12–14). Both types of molecules are expressed on activated T cells, with $CD4^+$ T cells displaying much higher levels than $CD8^+$ T cells. OX40L mRNA is expressed not only in lymphoid tissues but also in the heart, skeletal muscles, testes, and lungs (13). This should be related to the recent demonstration that human vascular endothelial cells constitutively express OX40L (15). The possible role of OX40L in extravascular migration of activated T cells is consistent with its relatively prolonged expression on activated T cells. Finally, it is of note that both OX40 and OX40L are expressed at high levels on HTLV-1-infected leukemic T cells and that OX40L expression on these cells is regulated by the tax gene of the retrovirus, suggesting a possible role of OX40/OX40L in this virus-induced leukemia (16–18).

As mentioned above, in vitro studies using OX40L transfectants have shown that this molecule provides a costimulatory signal to T cells, resulting in increased proliferation and cytokine production. To gain further insight into the possible physiologic significance of these findings we have examined the expression of OX40L on DC, the most potent APC for the induction of primary T cells responses (19, 20). We found that OX40L is constitutively expressed on mature human DC and that ligation of OX40L on monocyte-derived DC that are at an intermediate and reversible stage of maturation markedly enhances their development into typical mature DC.

Materials and Methods

Culture medium

The following culture media were used: RPMI 1640 medium (BioWhittaker, Walkersville, MA) supplemented with 100 IU penicillin, 2 mM glutamine, 100 μg/ml streptomycin, and serum-free HB101 medium (Irvine Scientific, Santa Ana, CA) supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 100 IU penicillin, and 100 μg/ml streptomycin.

Reagents

Recombinant human GM-CSF and IL-4 were provided by Dr. D. Bron (Institute Bordet, Brussels, Belgium) and Immunex Corp. (Seattle, WA), respectively. Recombinant human TNF-α and anti-TNF-α mAb were purchased from Genzyme (Cambridge, MA). The trimeric form of sCD40L was generated by Immunex Corp. Anti-OX40L/gp34 mAbs, 5A8 (IgG1), TAG34 (IgG1), and 8F4 (IgG1) were described previously (16, 17). $F(ab')_2$ of 5A8 were generated by the ImmunoPure $F(ab')_2$ preparation kit (Pierce Chemical Co., Rockford, IL). Anti-CD11b (LM2/1), anti-CD61 (A.P.3), and anti-CD58 (TS2/9) were obtained from American Type Culture Collection (Rockville, MD). Isotype-matched negative control mAb (mouse IgG1 and mouse IgG2a) were prepared in our laboratory. Fluorochrome- or biotin-conjugated mAbs against the following Ags were used for immunostaining: CD5 (Leu 1), CD19 (Leu 12), CD16 (Leu 11c), CD56 (Leu 19), and CD1a (Leu 6; Becton Dickinson, Mountain View, CA); HLA-DR (TDR31.1), HLA class I (3F10), CD3 (UCTH1), CD4 (QS4120), CD8 (UCTH4), CD14 (UCHM1), CD54 (15.2), CD80 (BB1), and CD86 (BU63; Ancell, London, Canada); and CD33 (WM53) and CD11c (B-ly6; PharMingen, Mississauga, Canada). Rat anti-CD115 mAb (2–4A5) and FITC-conjugated goat anti-rat IgG were purchased from Zymed Laboratories, Inc. (San Francisco, CA). Anti-CD83 mAb (HB-15a) was a gift from Dr. T. Tedder (Duke University, Durham, NC). Human CD40L-transfected L cells and anti-CD40 mAb (mAb 89) were provided by Dr. J. Banchereau (Schering-Plough, Dardilly, France).

Monocyte-derived DC

Monocytes were isolated from PBMC from healthy adult donors by cold aggregation and one cycle of E-rosetting as previously described (21). Enriched monocytes were incubated in six-well tissue culture plates (5×10^6 cells/well) in serum-free RPMI 1640. After 1 h of incubation, nonadherent cells were removed to deplete residual T, NK, and B cells. The adherent cells were cultured in 3 ml of RPMI/10% FCS (BioWhittaker) supplemented with 800 U/ml GM-CSF and 25 ng/ml IL-4. On day 4, the cultures were fed by removing 2 ml of supernatant and adding 2 ml of fresh medium containing either GM-CSF and IL-4 or GM-CSF and 30 ng (final concentration, 10 ng/ml) of TNF-α. After 7 days of culture, the cells were washed, and 5×10^5 cells were cultured in 1 ml of HB101 medium containing 10 μg/ml of polymyxin B (Sigma Chemical Co.-Aldrich Canada Ltd., Oakville, Canada) in a 48-well plate in the presence of various stimuli. For the determination of cytokine production and phenotypic analysis, the supernatants and the cells were collected after 20, 40, and 48 h.

DC purification from human peripheral blood and culture

DC were purified as previously described (22). Briefly, enriched monocytes were incubated with anti-CD11b mAb and anti-CD16 mAb, washed twice, and submitted to immunomagnetic depletion using a mixture of anti-mouse IgG beads and anti-CD19 beads (Dynal A.S., Oslo, Norway), according to the instruction manual. The resulting DC preparations consisted of $HLA\text{-}DR^+$ (mean, 96.1%; range, 92.4–98.9%) and contained no detectable $CD56^+$, $CD20^+$, or $CD3^+$ cells. The purified cells were cultured in culture medium in RPMI/10% FCS supplemented with 800 U/ml GM-CSF and 25 ng/ml IL-4. After 24 h of culture, the cells were washed, and 1.5×10^5 cells were cultured in 0.5 ml of RPMI/10% FCS containing 10 μg/ml polymyxin B in 5-ml Falcon tubes (Becton Dickinson) in the presence of various stimuli. The supernatants were collected after 40 h.

Flow cytometric analysis

Cells were stained for OX40L expression by a three-step procedure. Briefly, cells were sequentially incubated with normal human IgG (150 μg/ml), anti-OX40L/gp34 mAb (clone 5A8 or TAG34) or isotype-matched control mAb at 5 μg/ml, biotinylated goat anti-mouse IgG (Tago, Burlingame, CA), and phycoerythrin-labeled streptavidin (Ancell). Stained cells were analyzed with a FACSort (Becton Dickinson).

Cytokine determination

IL-12 p40 and IL-12 p75 were measured by two-site sandwich ELISA employing clones 2-4A1 and 20C2 as capture Ab, respectively; clone 4D6 conjugated to horseradish peroxidase was used as the second Ab. These mAbs were provided by Dr. M. Gately (Hoffmann-La Roche, Nutley, NJ). The sensitivity of the assay for IL-12 p40 and p75 was 12.5 pg/ml. TNF-α was measured by two-site sandwich ELISA as previously described (23). The limit of detection for the TNF-α ELISA was 150 pg/ml. IL-1β and IL-6 were measured with Quantikine ELISA kits (R&D Systems, Inc., Minneapolis, MN) according to the supplier's protocol.

Statistical analysis

A paired t test was used to determine the statistical significance of the data. Because of nonnormal distribution of samples, Wilcoxon's signed rank sum test was used to analyze the effect of anti-OX40L mAb on blood DC (bDC). A value of $p < 0.05$ was chosen for rejection of the null hypothesis.

Results

Expression of OX40L on monocyte-derived DC

In the first part of this study, the expression and the function of OX40L were examined in two types of monocyte-derived DC (Mo-DC) that were reported to be at different stage of maturation (24, 25). Immature dendritic-like cells were obtained by culturing peripheral blood monocytes for 7 days in IL-4- and GM-CSF-supplemented medium (these cells are referred to hereafter as IL-4-Mo-DC), more mature dendritic-like cells were obtained under identical conditions, except that TNF-α was added during the last 3 days of culture (TNF-Mo-DC). Initial experiments confirmed that TNF-Mo-DC expressed much more CD80, CD86, CD40, and CD54 than IL-4-Mo-DC, supporting that idea that they are more mature; moreover, TNF-Mo-DC, but not IL-4-Mo-DC, contained $CD83^+$ cells, a DC differentiation marker (26). None of the freshly prepared subsets of Mo-DC expressed OX40L as revealed by staining with either 5A8 or TAG34 mAbs (data not shown). Because ligation of CD40 was reported to up-regulate the expression of several surface molecules on DC, Mo-DC were stimulated with sCD40L and examined for OX40L expression (25, 27). As shown in Figure 1, CD40 stimulation induced OX40L on both types of Mo-DC; moreover, this effect was rapid (detected after 4 h) and prolonged (still present after 3 days).

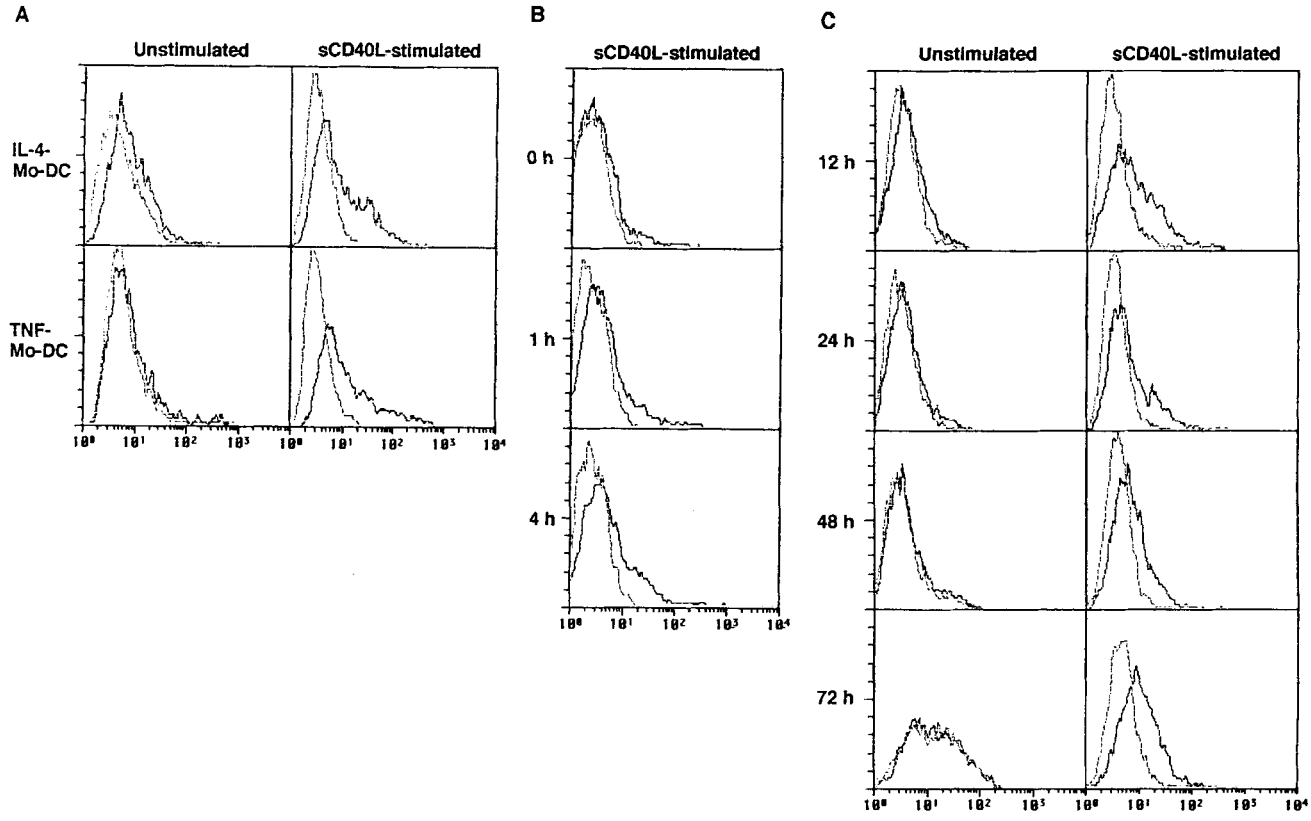

FIGURE 1. Expression of OX40L on monocyte-derived DC. Monocyte-derived DC were washed and resuspended in culture medium supplemented or not with sCD40L (1 μg/ml); cells were then stained with 5A8 mAb (dark lines) or control isotype matched Ab (faint lines). *A*, Expression of OX40L after 20 h of culture; *B* and *C*, expression of OX40L on TNF-Mo-DC after the indicated periods of stimulation. Similar results were obtained by staining the cells with TAG34 mAb recognizing a nonoverlapping epitope of OX40L. Each dataset is representative of at least three experiments.

Engagement of OX40L costimulates cytokine production by Mo-DC

To examine the idea that OX40L may signal activated mouse B cells for increased proliferation and Ig production, we examined whether ligation of OX40L may regulate cytokine production by Mo-DC. When cultured in HB101 serum-free medium alone or supplemented with anti-OX40L mAb, these cells released no or negligible amounts of IL-12 p40, TNF-α, IL-1β, or IL-6 (Fig. 2). Upon stimulation with sCD40L, the two subsets of Mo-DC released similar levels of IL-12 p40 and IL-1β; however, IL-4-Mo-DC produced much more TNF-α and IL-6 than TNF-α-Mo-DC. Ligation of OX40L markedly costimulated cytokine production by sCD40L-activated IL-4-Mo-DC; IL-12 production was increased 4-fold, TNF-α 23-fold, IL-1β 35-fold, and IL-6 4-fold. The enhancing effect of anti-OX40L mAb was much less pronounced on TNF-Mo-DC. The effects of anti-OX40L were specific inasmuch as they were not mimicked by mAbs binding to other molecules expressed on DC, such as CD58, CD11b, and CD61. Moreover, $F(ab')_2$ of anti-OX40L mAb displayed similar activity as intact mAb, ruling out an FcγR-mediated mechanism (Fig. 3). Thus, the OX40L molecules expressed on activated Mo-DC were functional and capable of transducing signals after cross-linking.

OX40L stimulation enhances the expression of costimulatory molecules on Mo-DC

To explore the above observations that OX40L-mediated signals enhanced cytokine productions by sCD40L-stimulated DC, we next examined whether OX40L ligation may also augment the expression of costimulatory molecules by sCD40L-stimulated DC. To this end, IL-4-Mo-DC were cultured as in the above experiments (with anti-OX40L mAb, sCD40L, or both) and stained after 2 days of culture with a panel of mAbs. As expected (25, 27), sCD40L alone was sufficient to up-regulate CD80, CD86, and CD54, whereas anti-OX40L mAb alone had no significant effect (data not shown). However, ligation of OX40L on sCD40L-stimulated cells further increased the expression of CD86, CD80, CD54, and CD40, without affecting that of HLA-DR or MHC class I molecules (Fig. 4). Ligation of OX40L on activated TNF-Mo-DC exerted a similar, but much less pronounced, effect (data not shown), presumably because these costimulatory molecules were already expressed at high levels before any stimulation.

Engagement of OX40L promotes the maturation of IL-4-Mo-DC

The above two series of experiments revealed that the main function of OX40L was to amplify the effects of sCD40L on cytokine production and on the expression of costimulatory molecules by immature IL-4-Mo-DC. Given that CD40 stimulation as well as TNF-α were shown to promote the maturation of DC, we next examined whether stimulation through OX40L could enhance the maturation of CD40L-activated IL-4-Mo-DC and whether this was TNF-α dependent. To this end, IL-4-Mo-DC were cultured for 40 h with sCD40L, anti-OX40L mAb, or both, and their maturation was evaluated by examining 1) the expression of CD83 and CD115 Ags known to be, respectively, up- and down-regulated on

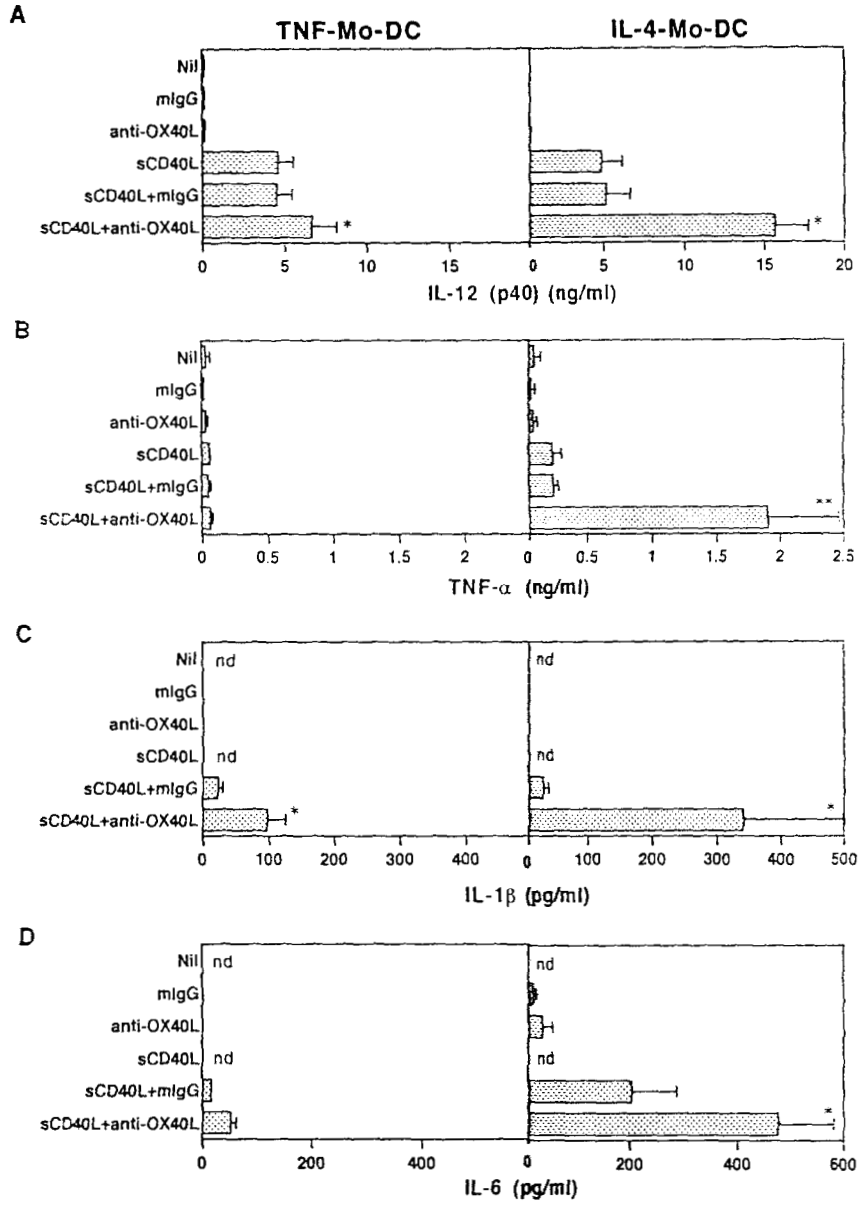

FIGURE 2. Engagement of OX40L costimulates cytokine production by activated Mo-DC. Mo-DC were cultured with or without sCD40L (1 μg/ml), anti-OX40L mAb (5A8, 5 μg/ml), or mouse IgG1, used alone or in combination. TNF-α was measured after 20 h of stimulation, and IL-12 p40, IL-1β, and IL-6 were measured after 48 h. The *left column* refers to TNF-Mo-DC, and the *right column* to IL-4-Mo-DC. Shown are the mean ± SEM of nine experiments. ** indicates $p < 0.01$; * indicates $p < 0.05$. nd, not done.

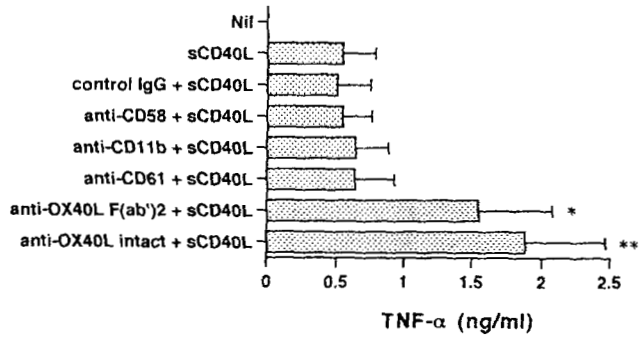

FIGURE 3. The activity of anti-OX40L mAb is Fcγ-independent. IL-4-Mo-DC were stimulated with sCD40L (1 μg/ml) in the presence of control IgG1, anti-CD58, anti-CD11b, anti-CD61, and anti-OX40L mAbs or F(ab')$_2$ anti-OX40L, all at 5 μg/ml. TNF-α was measured after 20 h of stimulation. Shown are the mean ± SEM of five experiments. ** indicates $p < 0.02$; * indicates $p < 0.05$.

mature DC (28–30) and 2) cellular morphology. As shown (Fig. 5), anti-OX40L mAb markedly amplified the sCD40L-induced down-regulation of CD115 and up-regulation of CD83; moreover, anti-OX40L mAb alone slightly, but consistently, affected CD115 and CD83 expression. When examined with reverse phase contrast microscope (Fig. 6), unstimulated IL-4-Mo-DC appeared as round nonaggregated cells, sCD40L-stimulated cells formed aggregates and displayed small dendrites, whereas anti-OX40L- and sCD40L-stimulated cells displayed multiple and very long dendrites, resulting in a stellate morphology. Again, stimulation with anti-OX40L mAb alone consistently provoked cell aggregation as well as some dendrite formation. Thus, OX40L was probably expressed at very low, but functionally sufficient, levels. To examine the role of endogenous TNF-α, IL-4-Mo-DC were stimulated as described above with sCD40L and anti-OX40 mAb in the presence of either anti-TNF-α or control IgG and examined for CD115 expression. As shown (Fig. 7), neutralization of TNF-α did not prevent the down-regulation of CD115 on stimulated cells, whereas it effectively inhibited the effect of exogenous TNF-α. Finally, it is of note that OX40L expression on sCD40L- and anti-OX40L mAb-stimulated cells remained stable after 96 h of culture in the absence of stimulation (data not shown), suggesting that this molecule may be constitutively expressed on differentiated DC.

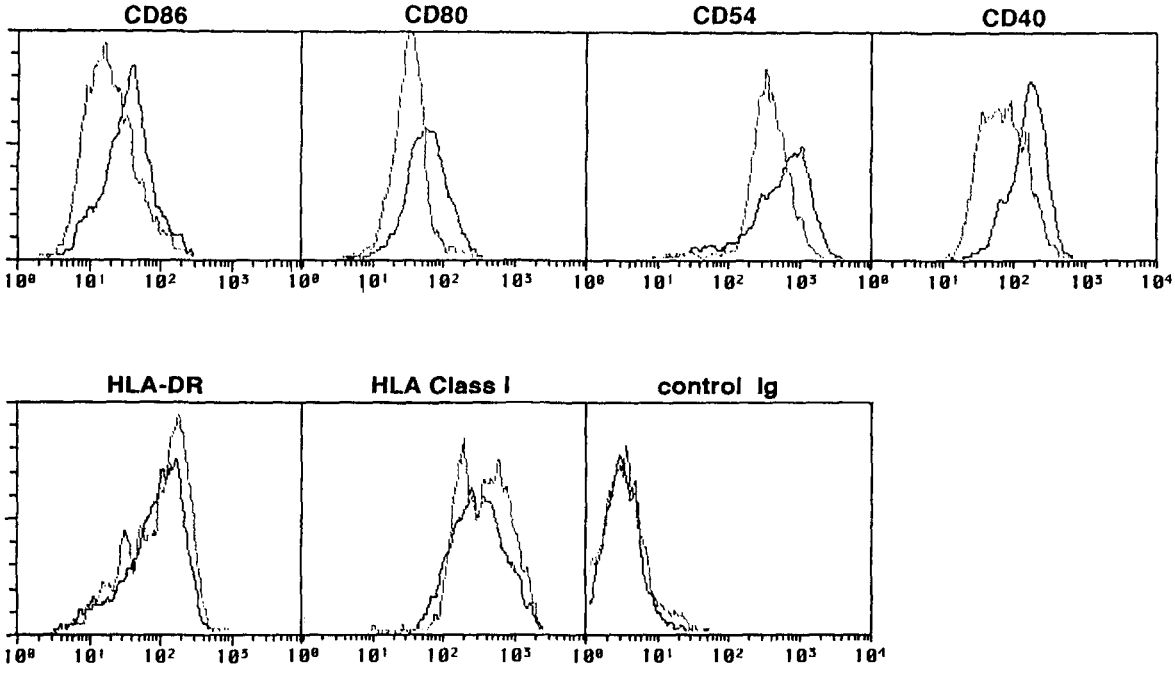

FIGURE 4. Engagement of OX40L enhances the expression of costimulatory molecules on activated IL-4-Mo-DC. IL-4-Mo-DC were cultured for 48 h with sCD40L (1 μg/ml) and anti-OX40L mAb (5A8, 5 μg/ml), washed, and stained with a panel of fluorochrome-conjugated mAbs. The histograms correspond to cells stimulated with either sCD40L plus anti-OX40L mAb (dark lines) or sCD40L plus normal mouse IgG1 (faint lines). The figure is representative of four experiments.

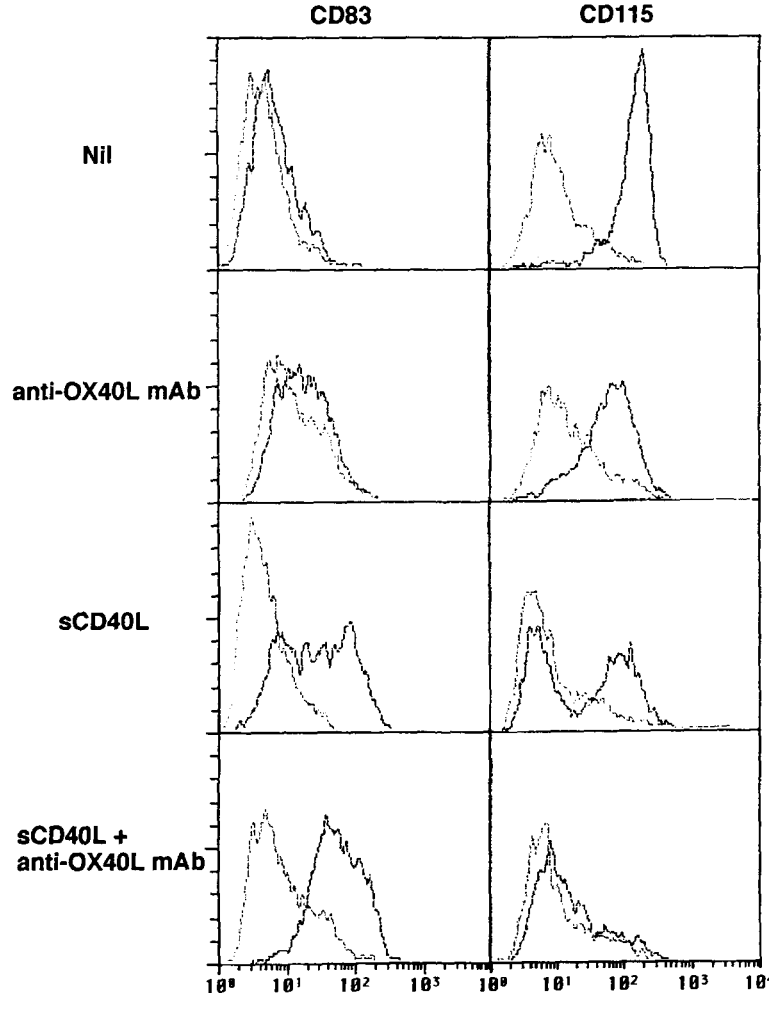

FIGURE 5. Engagement of OX40L enhances the maturation of activated IL-4-Mo-DC. IL-4-Mo-DC were cultured for 40 h with sCD40L (0.5 μg/ml), anti-OX40L mAb (5 μg/ml), or normal mouse IgG1, used alone or in combination. Staining with anti-CD115 and anti-CD83 mAbs is represented by dark lines; faint lines correspond to isotype-matched controls. The results are representative of four experiments.

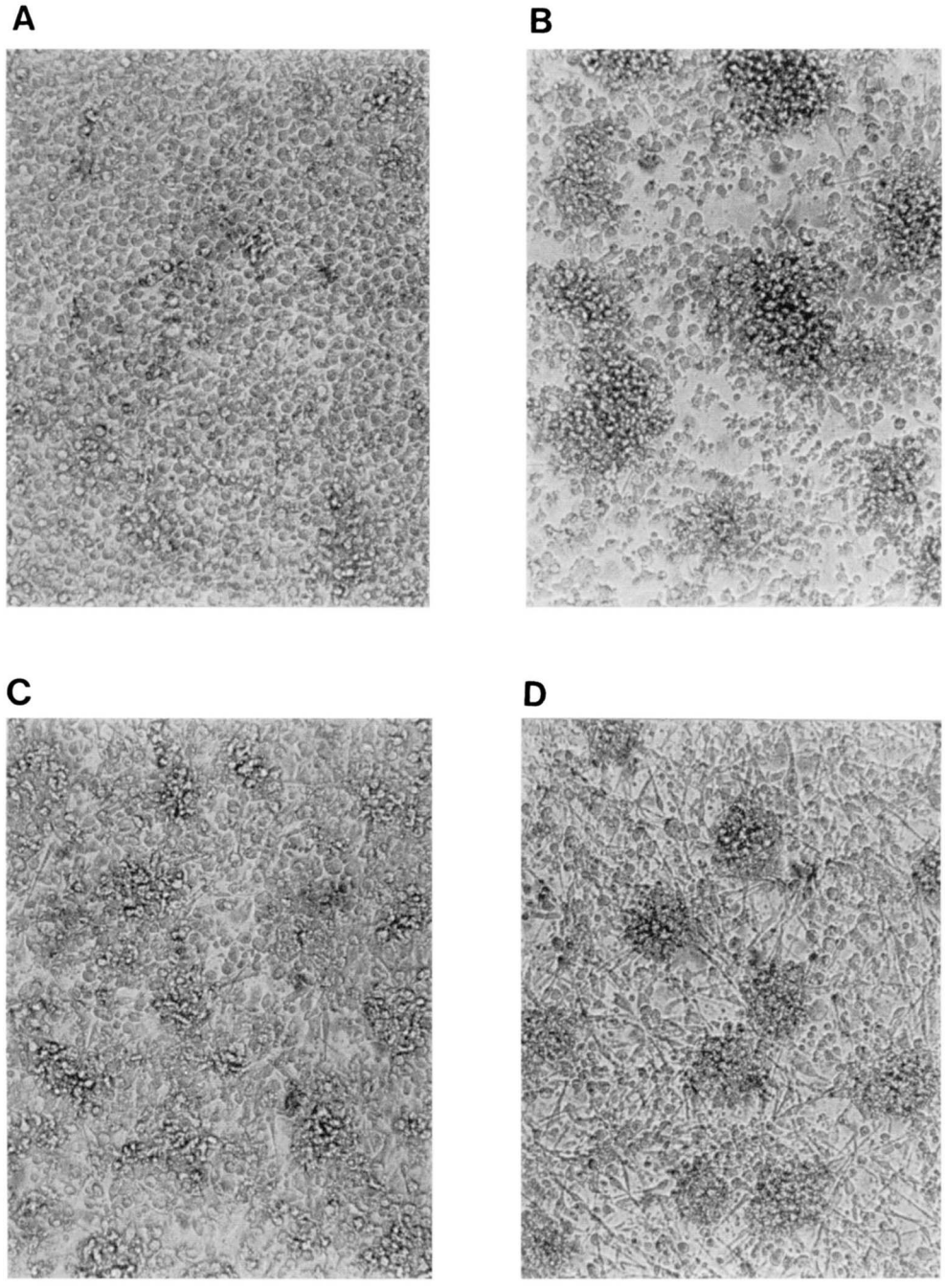

FIGURE 6. Engagement of OX40L alters the morphology of IL-4-Mo-DC. IL-4-Mo-DC were cultured for 20 h with control IgG (*A*), sCD40L (*B*), anti-OX40L mAb (*C*), or both (*D*) and examined under an inverted phase contrast microscope (original magnification, ×100).

Functional expression of OX40L on peripheral bDC

To determine whether OX40L expression was restricted to in vitro generated DC or whether it reflected a more general and physiologic property of DC, peripheral bDC were stained with anti-OX40L mAb. As shown in Figure 8*A*, freshly isolated DC constitutively expressed OX40L. After overnight culture, bDC lost surface OX40L and segregated into two subpopulations with distinct side scatter (SSC) properties (Fig. 8*B*). Interestingly, OX40L was spontaneously reexpressed on the high, but not on the low, SSC subset of bDC (Fig. 8*C*). Stimulation with sCD40L up-regulated OX40L on high SSC cells and induced its expression on the low SSC subset (Fig. 8*D*). The phenotype of the high SSC subset that spontaneously expressed OX40L was next characterized. As shown in Figure 9, these cells were brightly stained with mAbs to Ags known to be preferentially expressed on mature DC, i.e., CD83, CD11c, and CD33 (26, 31, 32). Compared with low SSC bDC, the high SSC bDC expressed more CD86, CD40, CD5, HLA-DR, and MHC class I molecules and less CD4. Both populations contained some $CD8^+$ but no $CD115^+$ cells. Collectively, these observations were taken to indicate that OX40L was constitutively expressed of mature bDC, whereas it was inducible (via CD40 stimulation) on more immature cells. To examine the functional activity of OX40L, freshly isolated bDC were stimulated with anti-OX40L mAb, sCD40L, or both, and the culture supernatants were collected for the measurement of IL-12. Since no or very

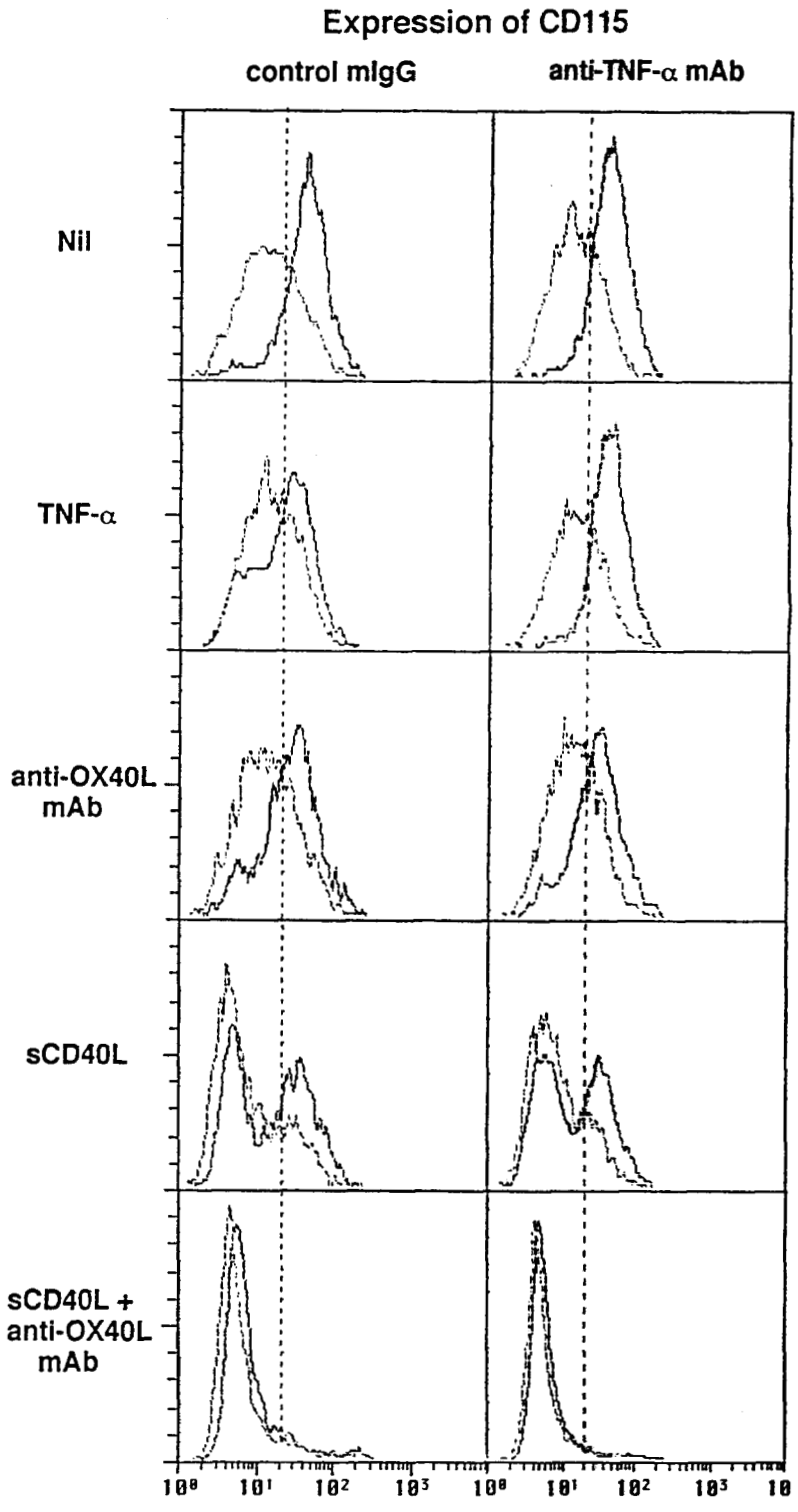

FIGURE 7. Role of endogenous TNF-α in the maturation of IL-4-Mo-DC. IL-4-Mo-DC were examined for CD115 expression after 40 h of culture in the presence of control IgG or anti-TNF-α mAb (each at 20 μg/ml); some cultures were stimulated with TNF-α (10 ng/ml), anti-OX40L mAb (5 μg/ml), sCD40L (0.5 μg/ml), or both. Similar results were obtained in two additional experiments.

little IL-12 p40 or p75 was induced under these conditions, we took advantage of a recent study showing that 24-h preincubation of bDC with GM-CSF and IL-4 markedly enhanced their production of IL-12 in response to stimulation with CD40L transfectants (33). Under these experimental conditions, significant levels of IL-12 p40 and p75 were released in response to CD40L stimulation, and these were slightly, but significantly ($p < 0.05$; $n = 5$), increased by costimulation with anti-OX40L mAb, indicating that OX40L was also functional on blood DC (Table I).

Discussion

In the present study we have demonstrated that DC are capable of expressing functional OX40L, a molecule previously shown to be expressed at high levels on activated mouse B cells and human vascular endothelial cells and at low levels on activated T cells (9, 13, 15). The expression and the function of OX40L were clearly related to the maturation stage of DC. First, it was constitutively expressed on differentiated DC, whereas it was observed only after stimulation of immature DC through CD40. Second, ligation of

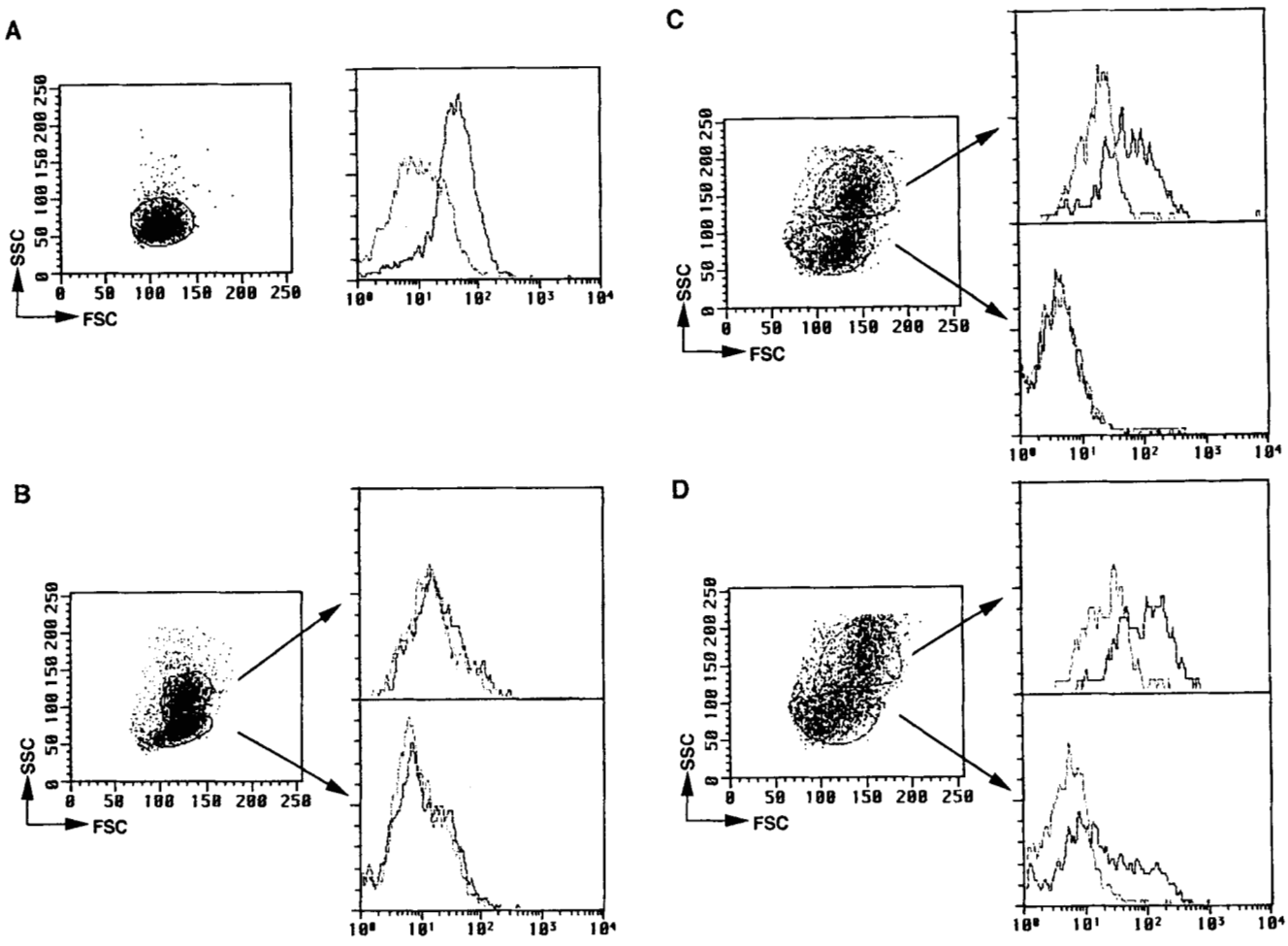

FIGURE 8. Expression of OX40L on purified blood DC. *A*, Freshly isolated bDC were stained with anti-OX40L mAb 5A8 (dark lines) or control IgG1 (faint lines). *B*, After overnight culture, bDC segregated into two subsets differing by SSC, neither of them expressed OX40L. *C*, Spontaneous re-expression of OX40L on the high SSC subset of DC after an additional 40 h of culture. *D*, DC were incubated overnight in culture medium alone and then stimulated for 40 h with sCD40L (1 μg/ml). Similar results were obtained by staining the cells with TAG34. The data are representative of four experiments.

OX40L had much more pronounced biological consequences on immature than on mature DC; in fact, engagement of OX40L markedly promoted the maturation of activated immature DC.

Previous studies have shown that engagement of OX40 on activated T cells by mAbs or OX40L transfectants significantly costimulates their proliferation and cytokine production, indicating that OX40L is a T cell costimulatory molecule (13, 14). The finding that OX40L is constitutively expressed by differentiated DC, the most efficient APC for Ag presentation to naive T cells, suggests that it may play a physiologic role in T cell priming. Consistent with this view is the observation that OX40 is expressed on activated naive human T cells with the same intensity and kinetics as on adult memory T cells (Y. Ohshima and G. Delespesse, unpublished observations). The role of OX40/OX40L interaction in naive T cell priming and Th subset development is under investigation. We observed that expression of OX40L in freshly isolated blood DC was down-regulated after overnight culture and that this molecule was spontaneously reexpressed after an additional 48-h culture. Moreover, these changes in OX40L expression by cultured DC were not influenced by exogenous GM-CSF or GM-CSF plus IL-4 (data not shown). Several mechanisms may account for OX40L down-regulation during the initial stage of culture, including selective death of OX40L-bearing cells, internalization, and shedding. The last possibility is favored by the observations that soluble OX40L is detectable in the supernatant fluids of OX40L transfectants, HTLV-1-transformed T cells, and HUVEC (14) (Y. Tanaka, unpublished observations). Moreover, the TNF-α cytokine is known to be generated as an active trimeric molecule by the proteolytic cleavage of its membrane-bound precursor (34). Finally, other members of the TNF family, including FasL and CD40L, were shown to be released from activated T cells in a soluble and biologically active form (35, 36). Regardless of its mechanism, the instability of membrane OX40L may render its detection difficult. This may explain the unexpected finding that anti-OX40L mAb consistently triggered immature IL-4-Mo-DC to aggregate and form dendrites despite the fact that OX40L could not be visualized on the surface of these cells. Peripheral bDC are known to be heterogeneous and to contain at least three subpopulations that are reportedly at different stages of maturation (31, 32, 37). Our preparations of DC were presumably comprised of two populations because the E-rosetting procedure used during their isolation is known to deplete one subset of blood DC (37). After overnight incubation, blood DC segregated into two distinct populations differing by their SSC properties and corresponding probably to mature and immature or precursor DC, as revealed by their differential expression of CD83, CD33, CD11c, CD5, CD86,

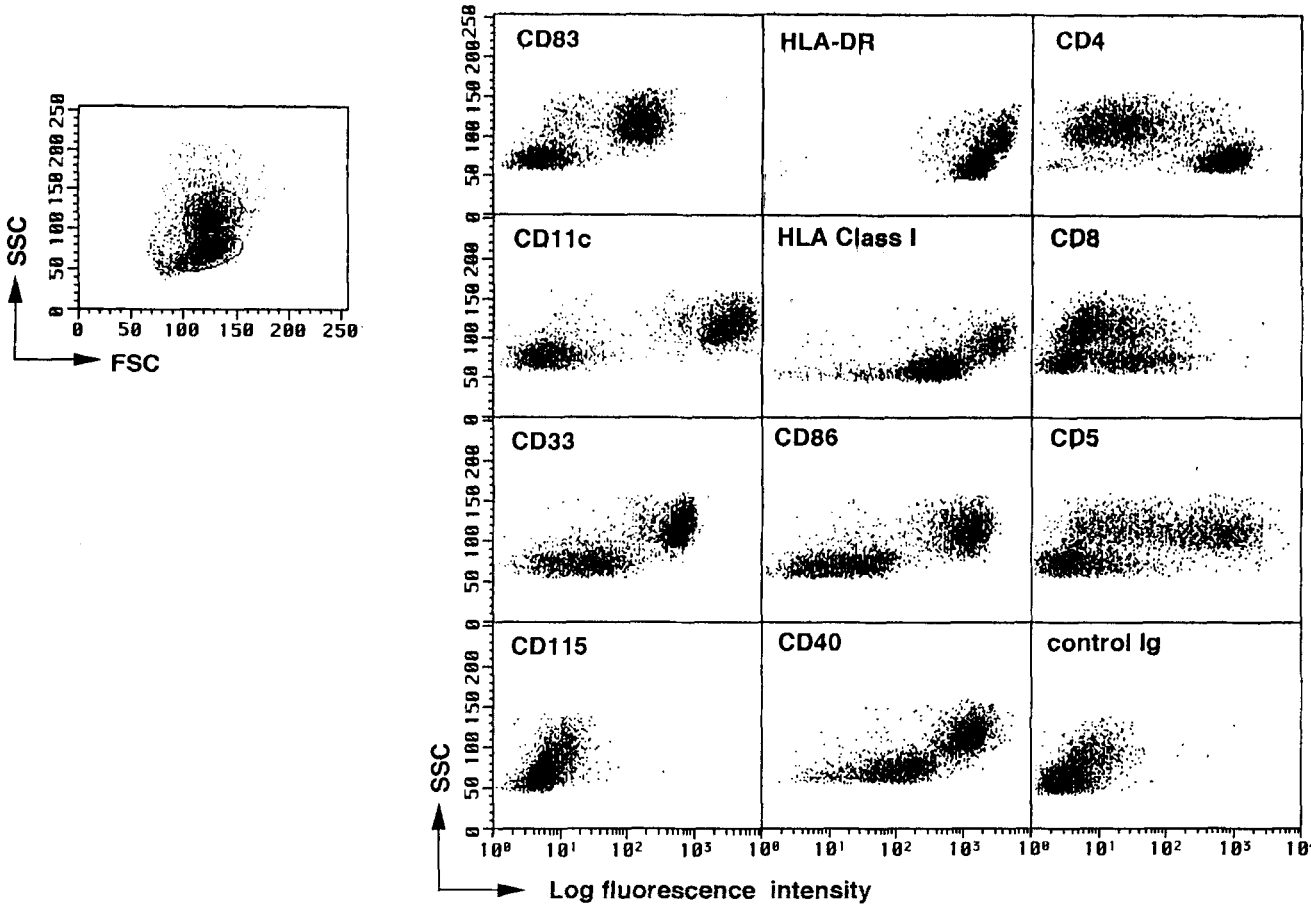

FIGURE 9. Phenotype of peripheral blood DC after overnight culture. Peripheral blood DC were cultured overnight and then stained with the indicated mAbs or isotype-matched controls. Cultured DC are comprised of two subsets differing by SSC (*left panel*); cells with high SSC express more CD83, CD11c, CD33, CD86, CD5, and CD40 (*right panel*). The results are representative of two experiments.

Table I. *Functional expression of OX40L on peripheral bDC* [a]

Addition to DC	Expt. 1		Expt. 2		Expt. 3	
	p40	p75	p40	p75	p40	p75
Nil	<	<	<	<	<	<
Control IgG	<	<	<	<	<	<
Anti-OX40L mAb	<	<	<	<	<	<
CD40L L cells	7.29	1.49	3.21	0.21	ND	ND
CD40L L cells + control IgG	6.62	1.33	3.07	0.21	0.55	0.17
CD40L L cells + anti-OX40L mAb	8.47 (1.28)	2.09 (1.57)	3.36 (1.09)	0.29 (1.31)	0.71 (1.28)	0.24 (1.36)

[a] Freshly isolated bDC were cultured with GM-CSF and IL-4 for 24 h, washed, and recultured for 40 h in the presence of CD40L-transfected L cells (3×10^4 cells), anti-OX40L mAb (5 μg/ml), or normal mouse IgG1, used alone or in combination. The enhancing effect of anti-OX40 mAb is shown in parentheses; three representative experiments of five. Results are expressed in ng/ml.

and CD40. The spontaneous re-expression of OX40L was observed only on mature DC, suggesting that similar to CD83, CD11c, CD33, and CD5, the OX40L may be a maturation marker of DC. Cross-linking of CD40 on DC is known to 1) up-regulate costimulatory molecules (e.g., B7.1, B7.2, and CD40), 2) trigger cytokine production (IL-12, IL-1β, macrophage inflammatory protein-1α, and IL-8), and 3) promote their maturation (27, 33). In agreement with this, we found that stimulation with sCD40L induced OX40L expression on precursor DC and up-regulated it on mature cells. Soluble CD40L stimulation also triggered IL-12 production, and most interestingly, this was slightly, but significantly, increased by OX40L ligation, indicating that this type II membrane protein had the capacity of transducing signals to DC. The signaling property of OX40L, which has been first demonstrated on mouse B cells (9), was much better evidenced on monocyte-derived immature dendritic-like cells obtained by culturing peripheral blood monocytes with IL-4 and GM-CSF. Several studies have shown that these IL-4-Mo-DC are at an intermediate stage of maturation. First, they express CSF-1R and can be converted to macrophages when cultured with M-CSF (28). Second, they are very efficient for Ag uptake and processing, but are poor T cell stimulators (25, 38). Third, according to some studies, they may still express residual CD14; moreover, they bear low levels of

costimulatory molecules and do not express CD83 (24). Although evidence was provided that TNF-α, IL-1, or CD40L stimulation promotes the maturation of IL-4-Mo-DC, more recent studies revealed that terminal differentiation requires exposure of these cells to monocyte-conditioned medium (24, 25, 29, 30, 38). Moreover, the activity of the conditioned medium is not ablated by neutralization of TNF-α, IL-1, IL-6, and IL-12 and cannot be replaced by the corresponding cytokines, suggesting that it is mediated by an unknown soluble factor(s). We found that ligation of OX40L on CD40L-activated IL-4-Mo-DC stimulated these cells to produce high levels of TNF-α, IL-12 p40, IL-1β, and IL-6 and to express more CD40, CD54, CD80, and CD86. Most remarkably, ligation of OX40L strongly enhanced their differentiation into $CD83^+$ $CD115^-$ cells displaying the typical stellate morphology of fully differentiated DC. This effect was largely TNF-α independent, since it was not affected by the neutralization of endogenous TNF-α. The phenotype of these cells remained stable, and they continued to express OX40L for as long as 96 h of culture in the absence of stimulant (data not shown), suggesting that similar to blood DC, mature Mo-DC constitutively expressed OX40L. Collectively, our data revealed that activation through both CD40 and OX40L was required for optimal differentiation of immature Mo-DC. Given that both OX40 and CD40L are expressed at high levels on activated T cells, these findings are likely to provide the molecular basis for the T cell-driven terminal differentiation of DC (13). This concept initially derived from in vitro experiments is also supported by some in vivo observations. In vitro, murine epidermal DC lines, displaying similar phenotypic and functional features of resident cutaneous Langerhans cells, undergo terminal differentiation upon Ag presentation to T cells. More specifically, these cells were shown to up-regulate CD86, lose surface CD115 (CSF-1R), and down-modulate their adhesive and phagocytic capacities. Similar to our findings, the T cell-driven maturation of murine DC was accompanied by IL-1β production (39–41). In vivo, Agger et al. (42) compared the phenotypic markers of splenic DC in the T cell area to those of DC in the marginal zone and concluded that the latter undergo terminal differentiation after migration into the T cell area.

Finally, it must be noted that DC were recently reported to express other members of the TNF family, including CD40L (43) and FasL (44). The latter has been found on a subset of CD8α-bearing murine DC and was shown to down-regulate the clonal expansion of primed CD4 but not CD8 T cells.

In conclusion, the present findings on the expression and functional activity of OX40L on DC suggest that OX40/OX40L interaction may regulate T cell priming by enhancing the activation and differentiation of both T cells and DC.

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