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Cross-Talk between Activated Human NK Cells and CD4^{+} T Cells via OX40-OX40 Ligand Interactions[1]

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It is important to understand which molecules are relevant for linking innate and adaptive immune cells. In this study, we show that OX40 ligand is selectively induced on IL-2, IL-12, or IL-15-activated human NK cells following stimulation through NKG2D, the low affinity receptor for IgG (CD16) or killer cell Ig-like receptor 2DS2. CD16-activated NK cells costimulate TCR-induced proliferation, and IFN-γ produced by autologous CD4^{+} T cells and this process is dependent upon expression of OX40 ligand and B7 by the activated NK cells. These findings suggest a novel and unexpected link between the natural and specific immune responses, providing direct evidence for cross-talk between human CD4^{+} T cells and NK receptor-activated NK cells. *The Journal of Immunology,* 2004, 173: 3716–3724.

For an effective T cell response at least two signals are needed: the first is delivered by TCR interaction with MHC and peptide, and the second involves ligation of co-stimulatory receptors. Costimulation can involve augmenting cell proliferation, cell survival, and/or the production of cytokines. Many receptors have now been described to be costimulatory, including receptors of the Ig superfamily, such as CD28 and ICOS, and receptors of the TNF superfamily. Interactions between TNF ligands and TNFR family members, including for example OX40 ligand (OX40L) and OX40, have been implicated in T cell co-stimulation (1). Expression of OX40L is inducible and has been reported on several hemopoietic cell types, including dendritic cells (2), B cells (3), T cells, and microglial cells, as well as on vascular endothelial cells (4). OX40L expression is induced on APCs several days after activation by CD40L-CD40 interactions or by inflammatory stimuli (1, 2). Recently, high levels of OX40L have been shown to be expressed on a new type of CD3⁻CD4⁺ accessory cell, located in B cell follicles, capable of promoting survival of Th2 cells through OX40-OX40L interactions (5). OX40 is expressed predominantly by activated CD4⁺ T cells (6). OX40⁺ cells are found in the T cell zones of lymphoid organs following priming with Ag (3), and also have been detected in situ in several inflammatory states, including experimental autoimmune encephalomyelitis, rheumatoid arthritis, chronic synovitis, graft-vs-host disease, and on tumor-infiltrating lymphocytes (6–9). Ligation of OX40 on CD4⁺ T cells by agonist reagents can increase clonal expansion and cytokine production (10), enhance memory T cell development (11), and augment anti-tumor immunity (12). OX40 has also been shown to play an important role in the stimulation of anti-viral CD4⁺ T cell responses in vivo (13).

NK cells are lymphocytes that provide innate immunity against tumors and virus-infected cells. A balance of signals received from multiple activating and inhibitory receptors regulates their effector functions (14). These receptors allow NK cells to rapidly survey their environment for danger. When an imbalance in signaling favors activation, secretion of cytokines and/or release of cytotoxic granules occurs (14). In humans, NKG2D is one of the activating receptors that is expressed on NK cells, γδ T cells, and CD8αβ T cells (15). NKG2D recognizes as ligands UL16-binding protein 1 (ULBP1), ULBP2, ULBP3, ULBP4, and the MHC class I chain-related molecules, MICA and MICB (15, 16). These NKG2D ligands are generally absent or expressed at low levels on most healthy cells, but can be induced by viral (17) and bacterial infections (18, 19). In addition, they are frequently up-regulated in many epithelial tumors (20) and in "stressed" cells (21).

Several studies have focused on the ability of NK cells to regulate adaptive immune responses through the production of Th1-type cytokines early during infection (22) or through the activation of dendritic cells (23). In addition, by establishing cocultures of NK- and Ag-activated T cells, it has been shown that human NK cells can be induced to secrete IFN-γ in response to IL-2 produced by activated T cells (24). In contrast, much less has been reported about the physical interactions that may take place between NK cells and adaptive immune cells, in particular CD4⁺ T cells.

In this study, we show that OX40L can be induced on human NK cells by stimulation through their activating NK receptors. In addition, we present direct evidence for cross-talk between CD4⁺ T cells and NK cells in which OX40-OX40L and CD28-B7 interactions contribute to T cell proliferation and IFN-γ production in response to TCR-induced activation.

Materials and Methods

Reagents, cytokines, Abs, and flow cytometry

Human rIL-12 and IL-15 were purchased from BioSource International (Camarillo, CA). The National Cancer Institute Biological Resources

Branch Preclinical Repository (Frederick, MD) generously provided human rIL-2. Staphylococcal enterotoxin B (SEB) and PHA were purchased from Sigma-Aldrich (St. Louis, MO). The following mouse anti-human mAbs were used: anti-killer cell Ig-like receptor (KIR)2DS2 (DX27), neutralizing anti-CD80 (L307), and anti-CD86 (IT2.2) (BD Pharmingen, San Diego, CA), FITC-conjugated anti-CD80 (BU63; Caltag Laboratories, Burlingame, CA), FITC-conjugated anti-CD86 (MEM-233; Caltag Laboratories), anti-CD8α (Leu2a; BD Pharmingen), anti-CD4 (Leu3a; BD Pharmingen), anti-HLA-DR (BD Pharmingen), anti-NKG2D (clone 149810; R&D Systems, Minneapolis, MN), anti-CD56 (DX32), neutralizing anti-OX40L (5A8) (2, 4), anti-CD16 (B73.1) (kindly provided by Dr. G. Trinchieri, Schering-Plough, Dardilly, France), and anti-CD3 (OKT3; American Tissue Culture Collection, Manassas, VA). PE-conjugated goat anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA), FITC-conjugated anti-mouse IgG was purchased from Zymed Laboratories (South San Francisco, CA), and goat anti-mouse IgG F(ab')$_2$ was from Cappel Laboratories (ICN Biomedicals, Opera, Milan, Italy). Cells were analyzed by using a FACSCalibur (BD Biosciences, San Jose, CA) or a small desktop Guava Personal Cytometer with Guava ViaCount and Guava Express software (Burlingame, CA). Viable lymphocyte populations were gated based on forward and side scatters and by propidium iodide staining.

Cell lines, plasmids, and transfectants

The NKL cell line, generously provided by Dr. Mike Robertson (25), was cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 200 U/ml human rIL-2. Cells were cultured at a density of 5×10^5/ml in a 37°C incubator with 5% CO_2. For all experiments, cells were grown at a density of 1×10^6/ml in medium containing IL-2. Generation of NKL stably expressing KIR2DS2 was described previously (26). Because mouse Ba/F3 pro-B cells are IL-3 dependent for their proliferation, the Ba/F3 cells used in these experiments were transfected with an expression plasmid containing the mouse cDNA IL-3 to provide for autocrine growth (kindly provided by Dr. S. Tangye, Centenary Institute, Sydney, Australia). MICA transfectants were established by retroviral transduction using the pMX-pie vector (27, 28) containing a MICA*0019 cDNA.

Preparation of NK cells and T cells

Small resting CD4$^+$ T lymphocytes were purified as follows: PBMC were isolated by lymphoprep density gradient centrifugation, monocytes and B cells were removed by adherence to nylon wool, then cells were labeled with anti-CD8, anti-CD56, anti-HLA-DR, and anti-CD19 mAbs, and these cells were mixed with magnetic beads coated with goat anti-mouse IgG (Dynal Biotech, Oslo, Norway). Thereafter, CD8$^+$, CD19$^+$, HLA-DR$^+$, and CD56$^+$ cells were removed by magnetic cell sorting. The remaining cells were >98% CD4$^+$CD3$^+$, as assessed by immunofluorescence and flow cytometric analysis. Polyclonal NK cell cultures were obtained by coculturing nylon nonadherent PBMC with irradiated (3000 rad) RPMI 8866 B cells for 9–10 days at 37°C in a humidified 5% CO_2 atmosphere, as previously described (29). NK cell cultures were >90% CD16$^+$CD56$^+$CD3$^-$, as assessed by immunofluorescence and flow cytometric analysis. Contaminating T cells were depleted by magnetic cell sorting, yielding a final NK population >98% CD16$^+$CD56$^+$CD3$^-$.

Stimulation of the cells, RNA preparation, microarrays, and data analysis

Twenty-four-well culture plates were coated with goat anti-mouse IgG (5 μg/ml, in carbonate buffer, pH 9.6) at 37°C for 4 h. Wells were washed three times with PBS and primary Abs were added to each well at 10 μg/ml, or amounts indicated in the figures, and incubated overnight at 4°C in PBS. When in combination with anti-NKG2D mAb, anti-KIR2DS2 mAb was used at 0.5 μg/ml. NKL cells were plated at 2×10^6/ml in each well in 500 μl of medium. Poly(A)$^+$ RNA was isolated using an mRNA isolation kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Gene expression modulation between unstimulated and stimulated NKL cells was evaluated by using Incyte standard procedures (Palo Alto, CA), as described elsewhere (30). Briefly, poly(A)$^+$ RNA were labeled with Cy3 or Cy5 fluorescent labeling dyes using reverse transcription, followed by hybridization onto a Human Drug Target 1 microarray (Incyte) (31, 32). This microarray contained a total of 9129 elements representing a total of 8481 unique gene clusters whose identity was confirmed by stringent PCR verification during manufacturing. The Cy3/Cy5 ratio for each element was considered valid if the signal to background ratios for both dyes exceeded 2.5, and if the signal of either dye exceeded 250 flu-

orescence units. A total of 6125 elements returned valid Cy3/Cy5 ratios for all 20 hybridizations (10 treatments hybridized in duplicates). Elements were further selected based on a minimum Cy3/Cy5 ratio of 2-fold in either direction in at least one experimental condition, yielding 406 elements of interest. These elements of interest were then clustered using an agglomerative clustering algorithm (Ward's method, JMP; SAS Institute, Cary, NC). All data are expressed in log$_2$, where negative values denote gene up-regulation (Cy3 < Cy5) and reciprocally, positive values represent gene down-regulation (Cy3 > Cy5).

Cytokine and proliferation assays

Homogeneous populations of cultured human primary NK cells were activated for 72 h with IL-2 (100 U/ml) and stimulated with anti-CD16 plate-bound mAb for 18 h. In some experiments, NK cells were preactivated with IL-15 (10 ng/ml) or IL-12 (10 U/ml). Dead cells were removed by Ficoll-gradient centrifugation. NK cells were fixed with 1% paraformaldehyde (in PBS, pH 7.4) for 7 min at room temperature. Different numbers of NK cells were plated with 1×10^5 highly purified autologous CD4$^+$ T cells, and cultured for 5 days in the presence of soluble anti-CD3 mAb (5 μg/ml) or SEB (0.5–25 ng/ml) or PHA (50 ng/ml). Blocking Ab against OX40L and/or CD80 and CD86 was added on day 0 at 5 μg/ml. Wells were pulsed with 0.5 μCi of [³H]thymidine for the final 18 h of culture, and incorporated radioactivity was measured in a scintillation counter. Data are represented as the mean of cpm ± SD (triplicates). In some experiments, supernatants were collected at day 3 or 5, and the amount of IL-4 and IFN-γ was quantified by specific ELISA kits (BioSource International).

Results

Microarray analysis shows up-regulation of OX40L following triggering of NK-activating receptors on a human NK cell line

Microarray analysis was used to characterize genes up-regulated by the stimulation of NKG2D alone or in combination with the DAP12-associated KIR2DS2-activating receptor. As a model, we used a human NK cell line, NKL, which constitutively expresses the DAP10-associated NKG2D receptor (33), and was transfected with KIR2DS2 (26). Because NKG2D alone is an insufficient stimulus for the transcription-dependent production of IFN-γ (26, 34), this cell system is particularly useful because it provided the opportunity to evaluate the efficacy of NKG2D costimulation using as a read out the amplification of KIR2DS2-induced IFN-γ (Ref. 26 and data not shown). Poly(A)$^+$ mRNA from resting and stimulated NKL cells was extracted, and cDNA was prepared for the comprehensive analysis of gene transcription by using microarray technology. A Human Drug Target 1 Incyte microarray containing a total of 9128 elements was used. Analysis of data was performed using a hierarchical clustering algorithm to group genes with similar expression patterns across all the samples. We focused our attention on a group of seven genes that were amplified significantly following the simultaneous cross-linking of KIR2DS2 and NKG2D receptors (Table I). These genes included three chemokines (i.e., lymphotactin, MIP-1β, and CCL18), granzymes B and H, the platelet-activating receptor homologue (a seven transmembrane receptor of unknown function), and the TNF member OX40L (CD134L). Among this group of genes, OX40L mRNA was the only one that was up-regulated by NKG2D cross-linking alone (Table I). Previously, OX40L expression has been implicated predominantly in the function of APCs, such as activated monocytes, dendritic cells, and B cells. Thus, this unexpected finding prompted us to investigate the role of OX40L in human NK cell function.

Results from the microarray experiment were confirmed by showing that cross-linking KIR2DS2, NKG2D, and KIR2DS2 plus NKG2D indeed enhanced transcription of OX40L in NKL cells, as determined by quantitative RT-PCR analysis (data not shown). More importantly, KIR2DS2- and NKG2D-induced activation resulted in an increased expression of OX40L on the cell surface of NKL cells, as determined by using a specific anti-OX40L mAb

Table 1. *Microarray analysis of NKL cells stimulated through NKG2D and/or KIR2DS2[a]*

Gene Name	Accession Number	cIg	KIR2DS2	NKG2D	KIR2DS2 + NKG2D
Lymphotactin	AL031736	0.31	−0.85	0.48	−2.39
MIP-1β	AV758471	0.06	−1.03	−0.07	−2.14
Granzyme H	NM__004131	0.43	−0.58	0.96	−1.87
Granzyme B	M57888	0.12	−0.48	0.81	−1.74
PAR	NM__013308	0.32	−0.72	0.07	−1.63
OX40L	**BE349175**	**0.13**	**−0.68**	**−0.20**	**−1.26**
CCL18	NM__00298	0.48	−0.58	0.14	−1.42

[a] Differential expression ratios of control Ig (cIg)-treated NKL cells (Cy3) compared to anti-KIR2DS2 and/or anti-NKG2D-treated NKL cells (Cy5) expressed in log$_2$. Negative values represent up-regulation of transcription compared with cIg-stimulated cells.

(Fig. 1*A*). Stimulation with high doses of anti-KIR mAb or anti-NKG2D mAb alone substantially up-regulated OX40L on the surface of NKL cells. In addition, anti-NKG2D mAb augmented up-regulation of OX40L on NKL cells stimulated with a suboptimal dose of anti-KIR mAb (Fig. 1*A*).

OX40 is expressed predominantly on activated CD4$^+$ T cells and prior studies have shown that interactions between OX40 on activated CD4$^+$ T cells and OX40L on APCs can augment T cell proliferation and cytokine production. Therefore, studies were performed to determine whether OX40L-bearing NK cells could co-stimulate CD4$^+$ T cell proliferation. NKL cells, which constitutively express OX40L (Fig. 1*A*), were cocultured with freshly isolated human CD4$^+$ T cells and were stimulated with anti-CD3 mAb or PHA. As shown in Fig. 1*B*, NKL indeed augmented CD4$^+$ T cell proliferation, and this activity was blocked, in part, in the presence of a neutralizing anti-OX40L mAb. These studies indicated that OX40L on NKL is functional and contributes to the proliferation of CD4$^+$ T cells. However, these studies were complicated by the necessity to use allogeneic CD4$^+$ T cells and also because NKL is a long-term NK cell line established from a patient with NK cell leukemia (25). Therefore, it was important to validate these findings using autologous NK cells and T cells from normal healthy individuals.

Both cytokines and NK receptor-mediated stimulation are required to induce OX40L on human peripheral blood NK cells

Freshly isolated, highly purified human peripheral blood NK cells do not express OX40L on the cell surface (data not shown), although a prior study had reported the presence of OX40L transcripts (35). Because the NKL cell line requires IL-2 for growth, we investigated whether OX40L could be induced on peripheral blood NK cells from healthy adults simply by culture in the presence of IL-2 or other cytokines known to stimulate NK cells, e.g., IL-12 and IL-15. As shown in Fig. 2*A*, culture of normal human peripheral blood NK cells in IL-2, IL-12, or IL-15 failed to induce OX40L. Therefore, based on the observation that OX40L was up-regulated in NKL cells stimulated through its activating receptors, we stimulated human polyclonal NK cells through CD16, an IgG FcR that signals via the ITAM-bearing FcϵRIγ and CD3ζ adapter proteins. Whereas treatment with cytokines alone failed to induce OX40L, the majority (typically 60% or more) of normal NK cells stimulated by plate-bound anti-CD16 mAb together with IL-2, IL-12, and IL-15 expressed OX40L at high levels on the cell surface (Fig. 2*A*). Stimulation with anti-CD16 mAb in the absence of IL-2 (or IL-12 or IL-15) induced OX40L only on a small proportion of NK cells. A dose-dependent induction of OX40L was observed when NK cells were activated with anti-CD16 mAb in the presence of IL-2 (Fig. 2*B*). In contrast to OX40L, culture of peripheral blood NK cells in IL-2 only did induce expression of CD86 (Fig. 2*C*) and this was not enhanced by stimulation with anti-CD16 mAb (Fig. 2*D*). CD80, another ligand of the CD28 costimulatory receptor on T cells, was not induced by IL-2 (Fig. 2*C*), and there was only a very slight indication of CD80 induction when both IL-2 and anti-CD16 stimulation were combined (Fig. 2*D*).

Because studies using the NKL cell line indicated that stimulation through the NKG2D receptor up-regulated OX40L, we also investigated this using peripheral blood NK cells from healthy adults. Polyclonal populations of NK cells from healthy individuals were expanded in culture, preactivated with IL-2 and stimulated with a plate-bound mAb against NKG2D. Fig. 3*A* shows that NKG2D cross-linking induced OX40L on ∼20% of the NK cells.

FIGURE 1. Up-regulation of OX40L on NKL by NK receptors and costimulation of CD4$^+$ T cell proliferation. *A*, NKL cells were stimulated with plate-bound mAb anti-NKG2D (10 μg/ml), anti-KIR2DS2 (10 μg/ml or 0.1 μg/ml), or both for 18 h. Cells were harvested and stained with PE-conjugated anti-OX40L mAb (open histograms) or with an isotype-matched cIg (filled histograms). *B*, Different amounts of paraformaldehyde-fixed NKL cells were cultured with 1 × 10^5 CD4$^+$ T cells in the presence of soluble anti-CD3 (5 μg/ml) or PHA (50 ng/ml). Neutralizing anti-OX40L mAb was added at day 0 and cocultures were harvested at day 5. Cultures were pulsed with 0.5 μCi of [^{3}H]thymidine for the final 18 h, and incorporated radioactivity was measured in a scintillation counter. A representative experiment of three is shown. Data are represented as the mean of cpm ± SD.
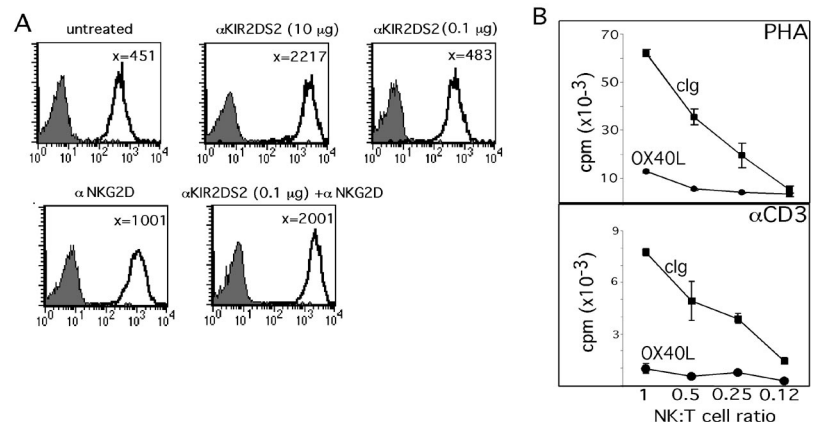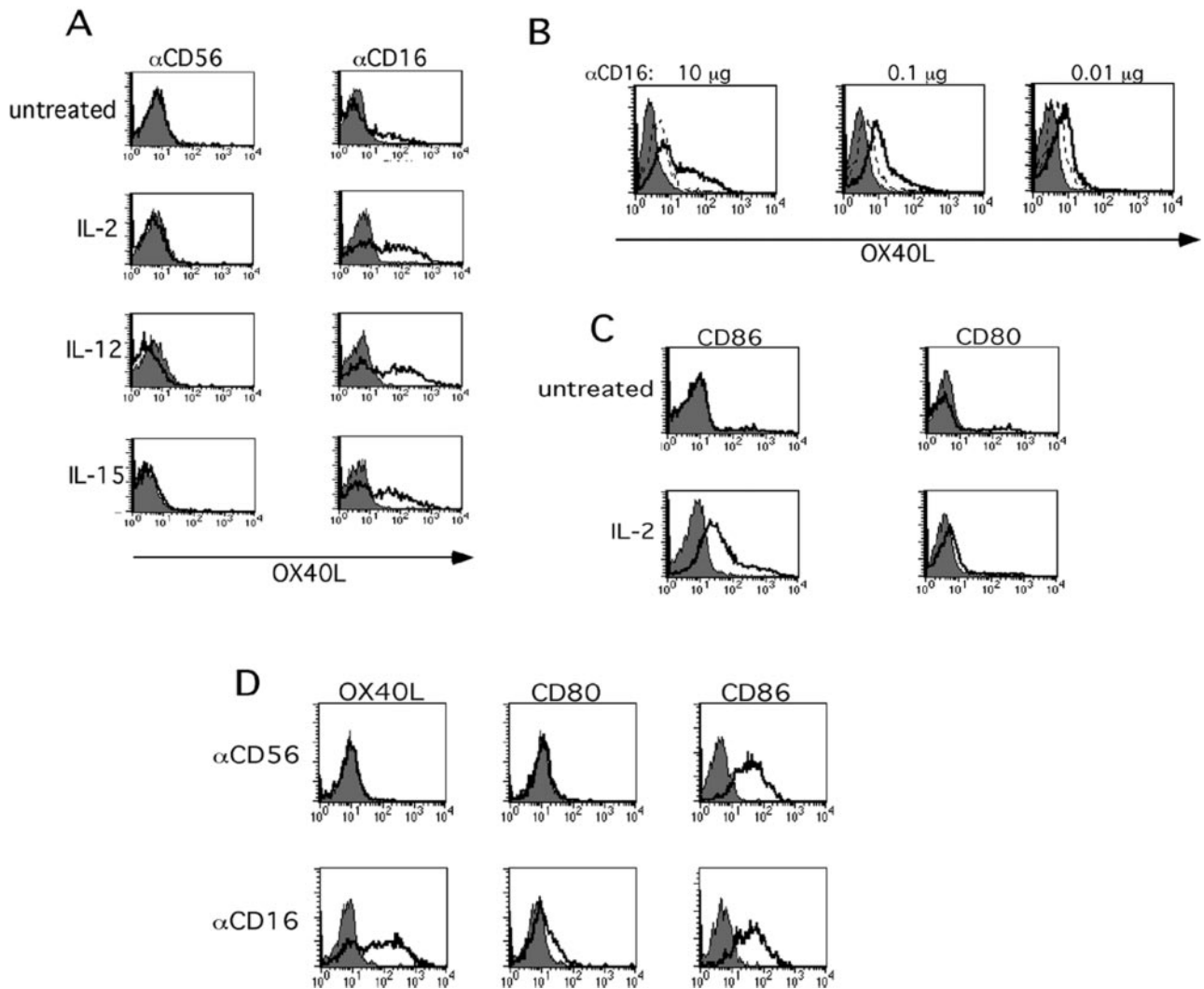
FIGURE 2. Induction of OX40L and B7 family members on human NK cells. *A*, Polyclonal human NK cells were preactivated with IL-2 (200 U/ml), IL-15 (10 ng/ml), or IL-12 (10 U/ml) for 48 h, and stimulated with plate-bound anti-CD16 mAb (saturating concentration). Plate-bound anti-CD56 mAb was used as a negative control. After 18 h of culture, cells were harvested and stained with PE-conjugated anti-OX40L mAb (thick line, open histograms) or with a cIg (filled histograms). *B*, IL-2-activated polyclonal NK cells were stimulated with different amounts (10 μg/ml, 0.1 μg/ml, and 0.01 μg/ml) of plate-bound anti-CD16 mAb (thick line). Plate-bound anti-CD56 mAb (dotted line) was used as a negative control for stimulation. Cells were harvested after 18 h and stained with FITC-conjugated anti-OX40L mAb (open histograms) or a cIg (filled histograms). A representative experiment of five is shown. *C*, Peripheral blood NK cells were cultured in the presence of IL-2 (200 U/ml) for 72 h and stained with FITC–conjugated anti-CD80 (thick lines, open histograms), FITC–conjugated anti-CD86 (thick lines, open histograms), or a cIg (filled histograms). *D*, IL-2-activated polyclonal NK cells were stimulated for 18 h with anti-CD16 or anti-CD56 (negative control) plate-bound mAbs. Cells were stained with PE-conjugated anti-OX40L mAb, FITC–conjugated anti-CD80 (thick lines, open histograms), FITC–conjugated anti-CD86 (thick lines, open histograms), or a cIg (filled histograms). In this experiment, CD86 was induced on these NK cells by coculture in IL-2, but was not further increased by stimulation with the anti-CD56 mAb used as a control.

As observed with anti-CD16 stimulation, induction of OX40L required both pretreatment with IL-2 and NKG2D activation because neither condition alone induced OX40L (data not shown). The ability of NKG2D stimulation to induce OX40L on NK cells was further validated by activation using stimulator cells bearing MICA, a physiological ligand of the NKG2D receptor. IL-2-preactivated peripheral blood NK cells were cocultured for 18 h with different ratios of the mouse pro-B cell line Ba/F3 or Ba/F3 cells stably expressing human MICA. As with anti-NKG2D mAb stimulation, OX40L was induced on ~20% of the IL-2-activated NK cells cocultured with MICA$^+$Ba/F3 cells, but not the untransfected Ba/F3 cells (Fig. 3*B*). Analysis of the kinetics of OX40L expression on human NK cells following stimulation with MICA-bearing cells showed that OX40L expression was transient; it was expressed rapidly after 5 h, peaked at 18 h, and then declined between 32 to 48 h poststimulation

(Fig. 3*C*). These IL-2-activated NK cells were able to efficiently kill the MICA$^+$Ba/F3 cells, but not the untransfected Ba/F3 cells, demonstrating that the NKG2D receptor on the NK cells was specifically activated (data not shown).

Therefore, both by stimulation with anti-NKG2D mAb and by interaction with MICA$^+$Ba/F3 cells, we observed induction of OX40L on a subset comprising ~20% of IL-2-activated peripheral blood NK cells (Fig. 3). An examination of the phenotype of the NK cells stimulated by either anti-NKG2D or MICA$^+$Ba/F3 cells revealed that OX40L was induced on both the CD56^{bright}CD16$^{-/}$$_{low}$ and on the CD56^{int}CD16^{high} peripheral blood NK cell subsets, although within these subsets a relatively higher fraction of the CD56^{bright}CD16$^{-/low}$ NK cells expressed OX40L (our unpublished observation). Therefore, the subset of peripheral blood NK cells presenting OX40L after NKG2D stimulation was not

FIGURE 3. NKG2D stimulation induces OX40L on human polyclonal NK cells. *A*, Polyclonal human NK were activated with IL-2 for 72 h and stimulated with plate-bound anti-NKG2D mAb (thick line). Anti-CD56 mAb was used as a negative control of stimulation (dotted line). After 18 h of culture, cells were harvested and stained with FITC-conjugated anti-OX40L mAb (thick line, open histogram) or with cIg (filled histogram). A representative experiment of six is shown. *B*, IL-2-activated polyclonal NK cells were cultured with different numbers of mock Ba/F3 (●) or human MICA⁺Ba/F3 (■) transfectants. After 18 h of coculture, NK cells were stained with anti-OX40L or control mAbs and the percentage of OX40L⁺ NK cells is shown. A representative experiment of three is shown. *C*, Kinetics of OX40L induction on NK cells by coculture with NKG2D ligand-bearing cells. IL-2-activated polyclonal NK cells were cultured at a 1:2.5 stimulator:NK cell ratio with mock Ba/F3 (*lower panels*) or MICA⁺Ba/F3 (*upper panels*) transfectants. Cells were harvested after 5, 18, 32, and 48 h of coculture, and stained anti-OX40L mAb (thick line, open histograms) and cIg (filled histograms). A representative experiment of three is shown.
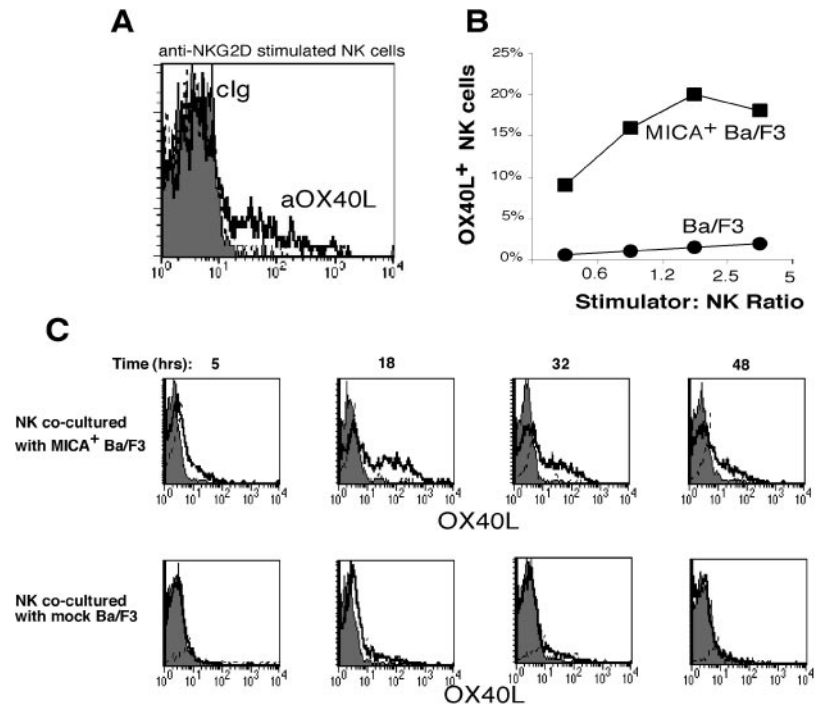
restricted to either of these functionally distinct subsets defined by levels of CD56 and CD16 expression. In experiments combining both anti-NKG2D and anti-CD16 mAb stimulation (using optimal and saturating concentrations of both mAbs), the proportion of peripheral blood NK cells that expressed OX40L was equivalent to using optimal stimulation with anti-CD16 alone (data not shown).

NK cell costimulation of TCR-dependent CD4⁺ T cell proliferation via OX40L-OX40 interactions

Our preliminary studies demonstrated that the OX40L⁺ NKL leukemic cells were able to augment the proliferation of allogeneic human resting peripheral blood CD4⁺ T cells stimulated with anti-CD3 mAb or PHA. The proliferation was partially, but substantially, inhibited by using a neutralizing anti-OX40L mAb (Fig. 1*B*). To address the potential interactions between NK cells CD4⁺ T cells in a more physiological context, we performed additional experiments using autologous NK cells and CD4⁺ T cells. We assayed proliferation induced not only by anti-CD3 mAb, but also by using autologous activated human NK cells (that express HLA-DR) to present SEB to autologous resting CD4⁺ T cells. Because we had determined that anti-CD16 was more efficient than anti-NKG2D for inducing OX40L on peripheral blood NK cells, this system was chosen to evaluate the role of OX40L in the interactions between NK cells and autologous CD4⁺ T cells. Highly purified, IL-2-preactivated peripheral blood NK cells were stimulated with anti-CD16 mAb, the NK cells were paraformaldehyde-fixed to prevent their proliferation or secretion of cytokines, and these cells were cocultured at varying ratios with highly purified resting autologous CD4⁺ T cells in the presence of soluble anti-CD3 mAb. As shown in Fig. 4*A*, CD16-activated autologous NK cells efficiently costimulated anti-CD3-induced proliferation of CD4⁺ T cells. This TCR-induced T cell proliferation was in part dependent upon OX40–OX40L interactions, because the proliferation was inhibited on average 60% (based on experiments using NK and T cells from seven different blood donors), in cultures containing the anti-OX40L specific neutralizing mAb 5A8. IL-2-activated NK cells that did not express OX40L were also able to costimulate the

anti-CD3-induced proliferation of autologous CD4⁺ T cells; however, this was always of a lower magnitude (approximately one third) than when the NK cells expressed OX40L as a consequence of prior stimulation via CD16 (Fig. 4*B*). An analysis of cytokines produced in these cultures revealed that the NK cell-costimulated T cells produced IFN-γ, but not IL-4 (Fig. 4*C*). Similar to the effects observed in the proliferation assays, anti-OX40L partially, but substantially, inhibited IFN-γ secretion induced by NK cell costimulation. In these experiments, fixed activated NK cells were used for costimulation to avoid the proliferation of the NK cells in response to IL-2, confirming that CD4⁺ T cells were the responding population in the cultures, and to exclude that NK cell-derived cytokines were required for costimulation. We also established autologous NK:T cell cocultures with irradiated NK cells, and similar to fixed activated NK cells, irradiated activated NK also efficiently costimulated T cell proliferation in a OX40–OX40L dependent manner (data not shown).

Next, we investigated the role of OX40-OX40L interactions in autologous NK:T cell cocultures in response to a physiological TCR ligand, rather than anti-CD3 mAb. Bacterial superantigens bind with high affinity to MHC class II Ags on APCs and with TCR β-chains on the responding T cells. This results in the T cell activation responsible for toxic shock syndrome and food poisoning. Activated NK cells express MHC class II molecules (36, 37) and present SEB to T lymphocytes (37). Thus, anti-CD16-activated MHC class II-positive NK cells and autologous freshly isolated resting CD4⁺ T cells were cultured in the presence of different concentrations of SEB. As shown in Fig. 5*A*, activated NK cells efficiently present SEB to autologous CD4⁺ T cells, stimulating T cell proliferation. Furthermore, OX40-OX40L interactions were required for optimal T cell proliferation, as shown in Fig. 5*B* by the ability of anti-OX40L mAb to substantially inhibit SEB-induced T cell proliferation. Collectively, these data indicate that CD16-activated NK cells can efficiently costimulate anti-CD3 or SEB-induced proliferation of autologous CD4⁺ T cells, and that OX40L-OX40 interactions are critically involved.

FIGURE 4. Anti-CD3 induced CD4^{+} T cell prolifer-
ation and IFN-γ production costimulated by OX40L on
autologous CD16-activated NK cells. *A*, IL-2-activated
polyclonal NK cells were stimulated with plate-bound
anti-CD16 mAb and fixed with 1% paraformaldehyde.
Different numbers of anti-CD16-activated NK cells
were plated with 1×10^5 autologous resting CD4^{+} T
cells in the presence of soluble anti-CD3 and cultured as
described in Fig. 1*B*. Neutralizing anti-OX40L mAb or
a cIg was added at day 0. A representative experiment of
four is shown. Data are represented as the mean of
cpm ± SD (triplicates). *B*, Cocultures of autologous ac-
tivated NK cells and resting CD4^{+} T cells at a ratio of
1:1 were established as described in Fig. 4*A*, using anti-
CD16-stimulated NK cells (NKS) or cIg (anti-CD56
mAb)-treated NK cells (NKNS). Neutralizing anti-
OX40L mAb or a cIg was added to the coculture of
NKS and autologous CD4^{+} T cells stimulated with anti-
CD3, as indicated. Data are represented as the mean of
cpm ± SE of seven independent experiments. *C*, Acti-
vated NK cell-resting CD4^{+} T cell cocultures stimulated
with anti-CD3 mAb were established as described in
Fig. 4*A*. Neutralizing anti-OX40L mAb or a cIg was
added to the cocultures, as indicated. Supernatants were
collected after 72 h and tested for the presence of IFN-γ
or IL-4. Data are represented as the mean ± SD (trip-
licates). A representative experiment of three is shown.
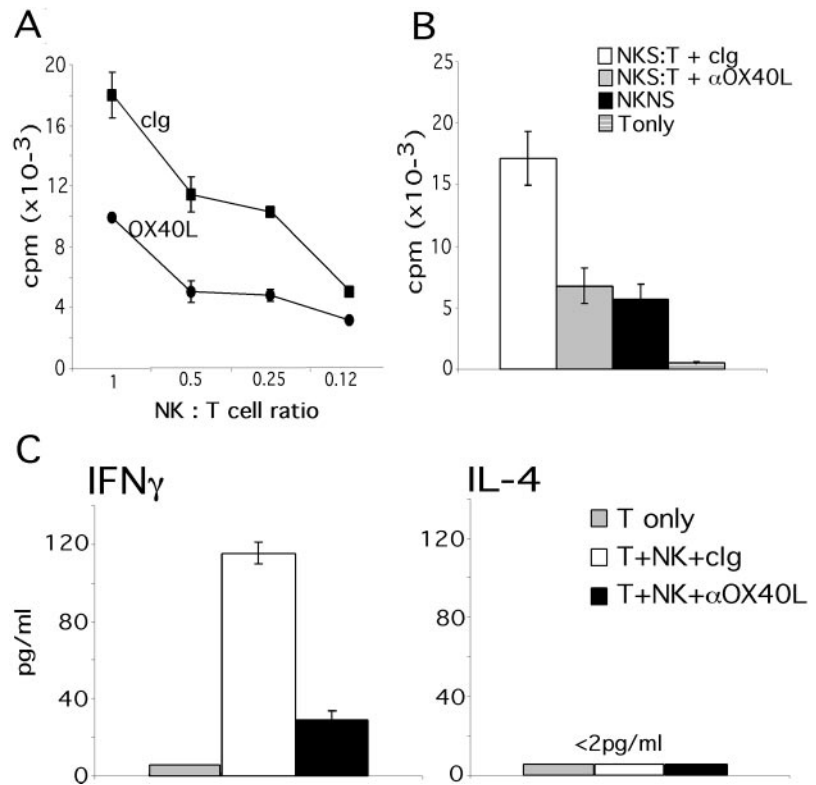
OX40L and B7 contribute to NK cell costimulation of CD4^{+} T cell

We considered that the inability of anti-OX40L mAb to com-
pletely block CD4^{+} T cell proliferation induced by activated NK
cells may be due to the presence of CD86 (and perhaps CD80) on
the activated NK cells (Fig. 2, *C* and *D*). Therefore, additional
experiments were performed in which CD16-stimulated NK cells
were cocultured with autologous CD4^{+} T cells and anti-CD3 using
a mixture of neutralizing mAbs against CD80 and CD86 (38) alone
or in combination with anti-OX40L (Fig. 6). Interestingly, while
mAbs against CD80 plus CD86 or OX40L individually partially
inhibited NK cell-induced T cell proliferation, we observed that
the combination of neutralizing mAbs against CD80, CD86, and
OX40L completely blocked TCR-dependent CD4^{+} T cell prolif-
eration (results from two different blood donors are shown and are
representative of five experiments). Collectively, these data show
that CD16-stimulated NK cells efficiently costimulate TCR-depen-
dent CD4^{+} T cell proliferation through the expression of OX40L
and B7-family members on the CD16-activated NK cells.

Discussion

Although it has been appreciated that NK cell production of IFN-γ
and possibly other cytokines and chemokines can affect innate and
adaptive immune responses, the potential role for direct cell-cell
interactions between NK cells and T lymphocytes, in particular
CD4^{+} T cells, has not been explored. Roncarolo and colleagues
(39) previously reported that human NK cell clones are able to
stimulate autologous CD4^{+} T cells, but the molecules involved in
this process were not defined. Our unexpected finding that OX40L
was up-regulated when NK cell receptors were stimulated on a
transformed NK cell line prompted us to re-evaluate how activated
NK cells are able to augment the TCR-dependent proliferation of
resting autologous peripheral blood CD4^{+} T cells. In this study,
we provide evidence that activated human NK cells are able to

help TCR-stimulated autologous CD4^{+} T cells by a process that
involves both OX40L and B7 costimulation.

Resting peripheral blood NK cells express neither OX40L nor
B7, and different stimuli are required to induce these costimulatory
molecules. Culture in IL-2 alone was sufficient to induce CD86,
but not OX40L. By contrast, stimulation with IL-2 and activation
through an NK receptor was required to induce OX40L. In addi-
tion to IL-2, IL-12 and IL-15 were also able to prime NK cells such
that they up-regulated OX40L when subsequently stimulated via
CD16. Because IL-12 and IL-15 are innate cytokines that may be
more available at a site of inflammation or an ongoing immune
response, these may represent the more physiologically relevant
cytokines in vivo.

With respect to the NK receptors that induced OX40L, our first
clues were derived from studies of the transformed NKL cell line.
Although this cell constitutively expressed OX40L, it can be up-
regulated by engaging either the DAP12-associated KIR2DS2 re-
ceptor that activates the Syk and ZAP70 tyrosine kinase pathways
(40), or by stimulating the DAP10-associated NKG2D receptor
that uses a PI3K-dependent activation pathway (33). We do not
have Abs that can discriminate between the activating and inhib-
itory KIR; therefore, in studies of peripheral blood NK cells, we
stimulated the NK cells with anti-CD16, which couples to the
ITAM-bearing FcεRIγ and CD3ζ adapter proteins and activates
Syk and ZAP70. When IL-2-preactivated peripheral blood NK
cells were stimulated with either anti-CD16 or anti-NKG2D (or
exposed to cells expressing the NKG2D ligand, MICA), OX40L
was rapidly induced. Interestingly, only a subset comprising
~20% of the peripheral blood NK cells expressed OX40L after
stimulating NKG2D, despite the fact that essentially all of the NK
cells expressed NKG2D. Further studies are needed to determine
why expression of OX40L was confined to a subset of the
NKG2D-activated NK cells. By contrast, a much larger frequency
of NK cells (typically 60% or more) expressed OX40L after CD16
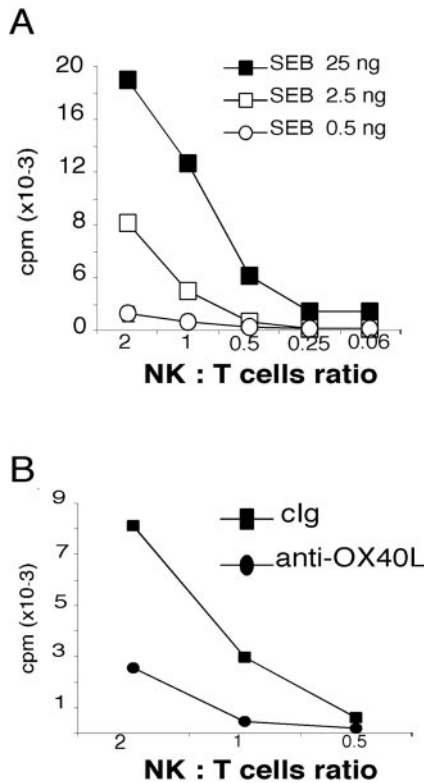
FIGURE 5. OX40L expressed on autologous NK receptor-activated NK cells is involved in SEB-induced proliferation of CD4^{+} T cells. *A*, Anti-CD16-activated NK cells were prepared as described in Fig. 4*A*. Autologous resting CD4^{+} T cells and activated NK cells were cocultured for 5 days in the presence of different concentrations of SEB, as indicated. Data are represented as the mean of cpm $\pm$ SD (triplicates). A representative experiment of two is shown. *B*, Autologous resting CD4^{+} T cells and anti-CD16-activated NK cells at the indicated ratios were cocultured in the presence of 2.5 ng/ml SEB for 5 days. Neutralizing anti-OX40L mAb or cIg was added at day 0. Data are represented as the mean of cpm $\pm$ SD (triplicates). A representative experiment of three is shown.

activation. Many of the NK receptors, e.g., NKp30, NKp44, NKp46, CD16, and the activating KIR (41), use ITAM-based adapter proteins to activate the Syk/ZAP70 tyrosine kinases. Therefore, we suspect that OX40L may be induced when any of these diverse receptors are engaged because they use a common downstream signaling pathway. Together with the ability of IL-2, IL-12, or IL-15 to render the NK cells permissive for NK receptor induction of OX40L, our findings indicate that OX40L may be

available in many different physiological situations for potential interactions with T cells bearing OX40.

Where might activated NK cells and CD4^{+} T cells interact? This interaction might happen in peripheral tissues such as the liver in which both NK cells and T cells are resident (42) and accumulate following virus infection (43). Furthermore, a recent report has revealed that NK cells are relatively abundant in the human secondary lymphoid organs (44), and importantly, immunohistochemistry studies have detected NK cells in the parafollicular T cell areas of human lymph nodes (24), providing another possible location in which NK:T cell interactions might occur during an immune response. During a viral or bacterial infection, NK cells in the lymph nodes may be exposed to an environment containing IL-2, IL-12, or IL-15, and potential NKG2D ligands or immune complexes (that engage CD16), thereby providing the stimuli needed for induction of OX40L and allowing them to interact with activated CD4^{+} T cell-expressing OX40.

It should be appreciated that activated human NK cells express high levels of MHC class II (36, 37), which provides them the potential to present Ag to human CD4^{+} T cells. Indeed, in these studies, we have shown that activated NK cells have the capability to directly stimulate CD4^{+} T cell proliferation by presenting SEB to CD4^{+} T cells. Therefore, activated human NK cells possess not only the required costimulatory molecules (e.g., OX40L and B7) for potential interaction with activated CD4^{+} cells, but they also, in theory, have the capacity of present Ags via MHC class II. Collectively, our in vitro experiments provide compelling evidence that human NK cells and autologous CD4^{+} cells can interact and that OX40L is an important participant in this process. It is difficult to provide formal proof of this interaction in vivo in humans. Unfortunately, because activated mouse NK cells (unlike human NK cells) do not express MHC class II, mice do not provide a relevant or appropriate model to examine MHC class II TCR-dependent CD4^{+} cell interactions with NK cells. Although dendritic cells are considered the most potent APCs, the fact that activated NK cells express MHC class II, CD86, and OX40L strongly suggests the possibility that they may also communicate directly with CD4^{+} cells. Otherwise, for what purpose would NK receptor-activated human NK cells express MHC class II, CD86, and OX40L?

Our findings demonstrate that human NK cell costimulation of TCR-induced CD4^{+} T proliferation depends in a large part on OX40–OX40L interactions. Studies conducted using OX40-deficient mice have shown that OX40-deficient CD4^{+} T cells initially become activated to secrete IL-2 (albeit at slightly lower levels than wild-type mice), but they are unable to sustain proliferation (45). Other studies performed on OX40^{−/−} mice reported that the impaired in vitro proliferative response to anti-CD3 stimulation

FIGURE 6. NK cell costimulation of TCR-dependent CD4^{+} T cell proliferation involves B7 family members. Cocultures of anti-CD16-stimulated autologous NK and CD4^{+} T cells at a ratio of 1:1 were established and stimulated with anti-CD3 as described in Fig. 4*A*. Neutralizing mAbs against CD80, CD86, and/or OX40L or cIg, as indicated, were added on day 0 at 5 μg/ml. Data are represented as the mean $\pm$ SD (triplicates). Two representative experiments of five are shown.
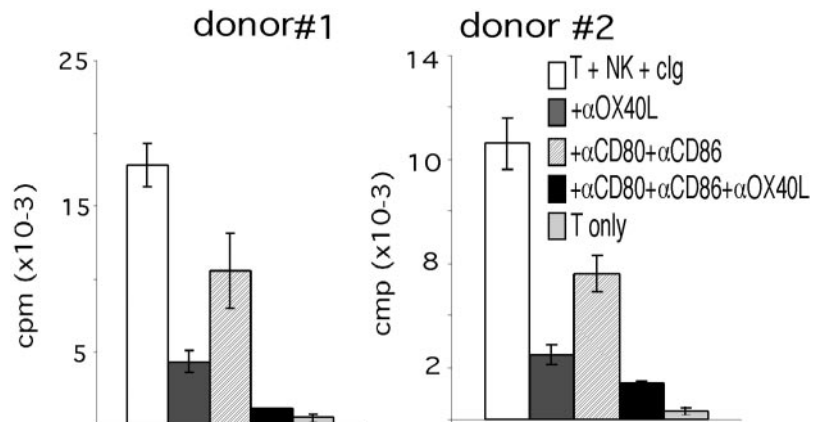
could not be corrected by the addition of exogenous rIL-2 (46). Most significantly, it has been shown that OX40 is a major regulator of anti-apoptotic proteins, such as Bcl-xL and Bcl-2 (45), and strongly promotes the survival of Ag-activated primary $CD4^+$ T cells (11). Similarly, the contribution of OX40-OX40L interactions to T cell proliferation that we have observed may favor T cell survival by the induction of Bcl-xL and Bcl-2, although this awaits further evaluation.

Previous studies reported that OX40L expressed on mouse B cells induce a Th2-type response, leading to the expansion of IL-4-producing mouse T effector cells and inhibiting IFN-γ expression (47, 48). In humans, a role for OX40L in the development of Th2 effector cells has also been reported (49). However, other studies do not support a differential role for OX40L in inducing Th1 vs Th2 differentiation (11, 13, 50, 51). suggesting that it only enhances the pre-existing response. In our studies using activated human NK cells to costimulate autologous $CD4^+$ T cells, we observed the production of IFN-γ, but not IL-4 secretion, by the TCR-activated T cells. These findings suggest that activated, mature human NK cells may preferentially promote T cell IFN-γ production.

We believe that the induction of OX40L on NK cells by NKG2D ligand-expressing cells might have important implications in the context of tumor surveillance and infectious diseases. It has been shown that the NKG2D ligand MICA is up-regulated on several human tumor cells and, interestingly, soluble MICA has been found in the serum of patients affected by different progressive tumors (52). In addition, several studies have reported that MICA is induced on cells infected with *Mycobacteria tuberculosis* (18), *Escherichia coli* (19), or cytomegalovirus (17). Thus, initial interactions between NK cells and NKG2D ligand-bearing cells or soluble NKG2D ligands may trigger killing and cytokine production and in the presence of IL-2, IL-15, or IL-12 may induce expression of OX40L on the NK cells. Subsequent interactions between $OX40L^+$ NK cells and $OX40^+$ T cells may amplify and sustain an adaptive ongoing immune response. At least under the experimental conditions used, we observed the induction of OX40L only on a subset of activated human peripheral blood NK cells. Further studies are necessary to resolve why some NK cells, but not others, expressed OX40L upon NKG2D stimulation, because all NK cells express NKG2D on the cell surface.

The OX40-OX40L interaction has been shown to induce bidirectional signals. For example, OX40L stimulation by OX40 transduces a signal in dendritic cells, which results in enhanced TNF-α and IL-1β production (2). Similarly, triggering of OX40L expressed on activated B cells results in B cell proliferation and Ig secretion (53). Finally, engagement of OX40L on vascular endothelial cells leads to the induction of c-*fos* and c-*jun* mRNA expression and the production of the chemokine RANTES (54, 55). Thus, while our present studies have focused on the potential role of OX40L on NK cell interactions with $CD4^+$ T cells, it will also be of interest to examine whether engagement of OX40L on NK cells might regulate their effector functions.

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