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J Immunol 2004; 173:3716-3724; doi: 10.4049/jimmunol.173.6.3716
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Cross-Talk between Activated Human NK Cells and CD4+ T Cells via OX40-OX40 Ligand Interactions

Alessandra Zingoni,*† Thierry Sornasse,‡ Benjamin G. Cocks,‡ Yuetsu Tanaka,§ Angela Santoni,† and Lewis L. Lanier*‡

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.

Abbreviations used in this paper: OX40L, OX40 ligand; cIg, control Ig; SEB, staphylococcal enterotoxin B; ULBP, UL16-binding protein; KIR, killer cell Ig-like receptor.

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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 HL-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 (KIR2DS2/DX27), neutralizing anti-CD80 (L507), 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-CD84 (Leu2a; BD Pharmingen), anti-CD4 (Leu3a; BD Pharmingen), anti-HLA-DR (BD Pharmingen), anti-NKG2D (clone H11032 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 scatter 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 × 10⁵/ml in a 37°C incubator with 5% CO₂. For all experiments, cells were grown at a density of 1 × 10⁶/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 dN32 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 removed by adherence to nylon wool, then cells were labeled with 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 scatter and by propidium iodide staining.

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 × 10⁶/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 was hybridized 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 fluorescence 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 log₂, where negative values denote gene up-regulation (Cy3 < Cy5) and reciprocally, positive values represent gene down-regulation (Cy3 > Cy5).

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-1ß, 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.

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 × 10⁶ 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 incorporation of 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).
Microarray analysis of NKL cells stimulated through NKG2D and/or KIR2DS2

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<th>Gene Name</th>
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<th>NKG2D</th>
<th>KIR2DS2 + NKG2D</th>
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</table>

* Different expression ratios of control Ig (cIg)-treated NKL cells (Cy3) compared to anti-KIR2DS2 and/or anti-NKG2D-treated NKL cells (Cy5) expressed in log2. Negative values represent up-regulation of transcription compared with cIg-stimulated cells.

(1A). 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. 1A).

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. 1A), were cocultured with freshly isolated human CD4+ T cells and were stimulated with anti-CD3 mAb or PHA. As shown in Fig. 1B, 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 NK 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. 2A, 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. 2A). 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. 2B). In contrast to OX40L, culture of peripheral blood NK cells in IL-2 only did induce expression of CD86 (Fig. 2C) and this was not enhanced by stimulation with anti-CD16 mAb (Fig. 2D). CD80, another ligand of the CD28 costimulatory receptor on T cells, was not induced by IL-2 (Fig. 2C), and there was only a very slight indication of CD80 induction when both IL-2 and anti-CD16 stimulation were combined (Fig. 2D).

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. 3A 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 co-stimulation 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 × 10⁶ 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 [3H]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.
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-activated 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. 3B). 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. 3C). 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 CD56brightCD16⁻/low and the CD56brightCD16bright peripheral blood NK cell subsets, although within these subsets a relatively higher fraction of the CD56brightCD16⁻/low NK cells expressed OX40L (our unpublished observation). Therefore, the subset of peripheral blood NK cells presenting OX40L after NKG2D stimulation was not
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. 1B). 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-activated 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. 4A, 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 OX40L-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. 1D). An analysis of cytokines produced in these cultures revealed that the NK cell-costimulated T cells produced IFN-γ, but not IL-4 (Fig. 4B). Similar to the effect 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 an OX40L-OX40L dependent manner (data not shown).

Next, we investigated the role of OX40L-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. 5A, activated NK cells efficiently present SEB to autologous CD4⁺ T cells, stimulating T cell proliferation. Furthermore, OX40L-OX40L interactions were required for optimal T cell proliferation, as shown in Fig. 5B 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-OX40L interactions are critically involved.
OX40L and B7 contribute to NK cell costimulation of CD4+ T cell

We considered that the inability of anti-OX40L mAb to completely 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 mAb (Fig. 4). 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 proliferation (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-dependent 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 addition 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 receptor 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 inhibitory 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-pretreated 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
NK AND T CELL CROSS-TALK VIA OX40-OX40L

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. 4A. 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 ± 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 ± 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, immunohistochimistry 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 of T cell.
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 T cells and inhibiting IFN-γ production (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 Mycobacterium 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 previous 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.

Acknowledgments
We thank Dr. Nigel Killeen and Dr. Cristina Cerboni for helpful discussion.

References


