

Inflammasome Reporter Cells

All you have to do is ASC

InvivoGen



NK Cells Stimulate Proliferation of T and NK Cells through 2B4/CD48 Interactions

Erika Assarsson, Taku Kambayashi, John D. Schatzle, Sven O. Cramer, Arne von Bonin, Peter E. Jensen, Hans-Gustaf Ljunggren and Benedict J. Chambers

This information is current as of July 19, 2018.

J Immunol 2004; 173:174-180; ;
doi: 10.4049/jimmunol.173.1.174
<http://www.jimmunol.org/content/173/1/174>

References This article **cites 52 articles**, 22 of which you can access for free at:
<http://www.jimmunol.org/content/173/1/174.full#ref-list-1>

Why *The JI*? [Submit online.](#)

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at:
<http://jimmunol.org/subscription>

Permissions Submit copyright permission requests at:
<http://www.aai.org/About/Publications/JI/copyright.html>

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
<http://jimmunol.org/alerts>



NK Cells Stimulate Proliferation of T and NK Cells through 2B4/CD48 Interactions¹

Erika Assarsson,* Taku Kambayashi,[†] John D. Schatzle,[‡] Sven O. Cramer,[§] Arne von Bonin,[§] Peter E. Jensen,[†] Hans-Gustaf Ljunggren,^{2*} and Benedict J. Chambers*

Few studies have addressed the consequences of physical interactions between NK and T cells, as well as physical interactions among NK cells themselves. We show in this study that NK cells can enhance T cell activation and proliferation in response to CD3 cross-linking and specific Ag through interactions between 2B4 (CD244) on NK cells and CD48 on T cells. Furthermore, 2B4/CD48 interactions between NK cells also enhanced proliferation of NK cells in response to IL-2. Overall, these results suggest that NK cells augment the proliferation of neighboring T and NK cells through direct cell-cell contact. These results provide new insights into NK cell-mediated control of innate and adaptive immunity and demonstrate that receptor/ligand-specific cross talk between lymphocytes may occur in settings other than T-B cell or T-T cell interactions. *The Journal of Immunology*, 2004, 173: 174–180.

Natural killer cells represent a third subset of lymphocytes important in the early defense against certain viruses, microbial infections, and cancer (1–5). NK cells have potent cytotoxic functions and are efficient producers of several cytokines, including IFN- γ , TNF- α , and GM-CSF (6). These cytokines provide important immunoregulatory properties by which NK cells can affect antimicrobial and antitumor responses as well as influence the outcome of autoimmune and hypersensitivity reactions (7). NK cells have also been reported to affect CTL responses in infection and tumor models as well as B cell responses (8, 9).

A number of receptor/ligand pairs have been shown to modulate lymphocyte responses. Among these are members of the CD2 subset of the Ig family of receptors, including CD2, CD48, CD58, CD84, signaling lymphocyte activation molecule, 2B4, and Ly-9. These receptors have been implicated in modulation of T or NK cell responses (10). One of the better characterized interactions is the one occurring between CD2 and CD48 that lowers quantitative thresholds and fine-tunes T cell activation both in vitro and in vivo (11–14). The 2B4 (CD244) is a cell surface glycoprotein that is expressed on all NK cells, $\gamma\delta$ T cells, and a subset of CD8⁺ T cells with memory/effector phenotype (15–19). Like CD2, 2B4 binds to CD48, but with 6- to 9-fold greater affinity (20–22). Engagement of 2B4 on NK cells with specific Ab leads to secretion of IFN- γ and increased cytotoxicity (15, 21, 23, 24).

Recent data have indicated a direct role for T-T cell interactions in the enhancement of Ag-specific proliferation of naive CD8⁺ T cells and cytokine-driven proliferation of memory CD8⁺ T cells (19, 25). Although the cytokine milieu formed by NK cells during, for example, an infection plays a central role in influencing the outcome of specific T cell-mediated immune responses, it is still unclear whether direct cellular interactions between NK and T cells can influence T cell responses. In the present study, we demonstrate that NK cells efficiently enhance CD4⁺ as well as CD8⁺ T cell proliferation and that this process is dependent upon interactions between 2B4 on NK cells and CD48 on T cells. In addition, we demonstrate that 2B4/CD48 interactions enhance NK cell proliferation in response to IL-2. These results represent a novel mechanism by which NK cells may bridge the innate and the adaptive immune systems and highlight the role for lymphocyte-lymphocyte interactions in driving immune responses.

Materials and Methods

Mice

The strains used in this study include adult C57BL/6 (B6), F5 (TCR transgenic (Tg)³ specific for an influenza nucleoprotein epitope, ASNENMDAM, presented on H-2D^b) (26), DO11.10 (TCR-Tg specific for an OVA epitope, ISQAVHAAHAEINEAGR, presented on I-A^d) (27), RAG-1^{-/-} (28), perforin-deficient (PKO)^{-/-}xRAG-1^{-/-} (29), B10.D2, CD48^{-/-} (30), and DBA/2. All animals were bred and housed under standard conditions at the Microbiology and Tumor Biology Center, Karolinska Institutet; Department of Pathology, Emory University School of Medicine; and Department of Pathology, Brigham and Women Hospital, Harvard Medical School (Boston, MA). All animal procedures were approved by the local departments as well as by the Committee for Animal Ethics in Stockholm, Sweden.

Cell lines and reagents

P815 tumor cells were transfected with 2B4 (clone P815-2B4.D1 used, designated P815-2B4; see below) and with H-2K^b (designated P815-K^b) and cultured in RPMI 1640, 5% FCS, 100 U/ml penicillin, and 100 U/ml streptomycin. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise specified. OVA-derived peptide (OVA_{323–339}), ISQAVHAAHAEINEAGR, was purchased from Interactiva (Ulm, Germany), and the influenza virus nucleoprotein-derived peptide (NP_{366–374}), ASNENMDAM, was purchased from Research Genetics (Huntsville, AL).

*Center for Infectious Medicine, Department of Medicine, Karolinska Institutet, Huddinge University Hospital, Stockholm, Sweden; [†]Emory University School of Medicine, Department of Pathology, Atlanta, GA 30322; [‡]Department of Pathology and Graduate Program in Immunology, University of Texas, Southwestern Medical Center, Dallas, TX 75390; and [§]Bernhard-Nocht-Institute for Tropical Medicine, Bernhard-Nochtstrasse, Hamburg, Germany

Received for publication February 20, 2003. Accepted for publication April 19, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by the Swedish Foundation for Strategic Research, the Karolinska Institutet, the Swedish Research Council, the Swedish Cancer Society, the Tobias Foundation, the Åke Wiberg Foundation, the Alex and Eva Wallström Foundation, the Lars Hiertas Foundation, the Swedish Foundation for International Cooperation in Research and Higher Education, and the Swedish Society for Medical Research.

² Address correspondence and reprint requests to Dr. Hans-Gustaf Ljunggren, Center for Infectious Medicine, F59, Department of Medicine, Karolinska Institutet, Huddinge University Hospital, SE-141 86 Stockholm, Sweden. E-mail address: hans-gustaf.ljunggren@medhs.ki.se

³ Abbreviations used in this paper: Tg, transgenic; DC, dendritic cell; FasL, Fas ligand; wt, wild type.

All Ab used were purchased from BD PharMingen (San Diego, CA), except for FITC anti-human Ig and human Ig (Jackson ImmunoResearch Laboratories, West Grove, PA); anti-murine CD3 (145-2C11) was purified from cell culture medium. All Ab used in proliferation assays were azide free.

Transfection

Tumor cells were grown to a density of 1×10^6 /ml, and $\sim 1 \times 10^7$ total cells were used for transfection. Cells were harvested and washed twice with serum and antibiotic-free medium. Approximately 10 μ g of plasmid (pBSRaEN with or without 2B4-D1 cDNA inserted) was linearized and used for transfection by electroporation at 960 μ F and 250 V. Cells were kept on ice for 15 min following transfection, then placed into complete medium (RPMI 1640, 10% FCS, 100 U/ml penicillin, 100 U/ml streptomycin) overnight at 37°C, 5% CO₂. The following day, cells were harvested and resuspended in complete medium containing 1 mg/ml G418 and plated into a single 96-well flat-bottom plate. Clones were obtained ~ 2 wk later and screened for 2B4 expression by flow cytometry.

Flow cytometry

Fc receptors were blocked by incubating the cells in anti-CD16/32 Ab at 10 μ g/ml, after which the cells were stained with the specified Ab or its isotype control at 10 μ g/ml. For stainings with Ig fusion proteins, splenocytes were stained with polyclonal human Ig (Jackson ImmunoResearch Laboratories) or 2B4 Ig (see below) at 10 μ g/ml, washed twice, and stained with FITC-conjugated goat anti-human Ig at 10 μ g/ml. The intensity of fluorescence was measured on a FACScan flow cytometer (BD Biosciences, Mountain View, CA) and analyzed using CellQuest computer software (BD Biosciences).

Cell purification

Murine CD4⁺, CD8⁺, and DX5⁺ cells were purified from spleens of mice using the MACS separation system (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's guidelines, and resuspended in complete α MEM (α MEM, 10 mM HEPES, 2×10^{-5} M 2-ME, 10% FCS, 100 U/ml penicillin, 100 U/ml streptomycin). Unless noted elsewhere, NK cells were purified from RAG-1^{-/-} mice. Purity of the NK cells was examined by flow cytometry by confirming the expression of NK1.1 and the lack of CD14 and MHC class II.

NK cell activation

For IL-2 activation, freshly purified DX5⁺ cells were cultured in complete α MEM with IL-2 at 1000 U/ml for 6 days. When not else noted, all NK cells used in this study were IL-2 activated.

Proliferation assays

Dendritic cells (DC) were prepared from bone marrow of mice, cultured for 6 days, as previously described (31), pulsed with ASNENMDAM or ISQAVHAAHAEINEAGR peptide for 24 h, and seeded at 2×10^4 cells/well in U-bottom 96-well plates. A total of 3×10^4 naive CD8⁺ or CD4⁺ TCR-Tg T cells and 4×10^4 DX5⁺ IL-2-activated NK cells were added to each well with the indicated Ab. In the DC-free cultures, 3×10^4 naive CD8⁺ or CD4⁺ B6 T cells were added to each well in a flat-bottom 96-well plate precoated with anti-CD3 Ab at 10 μ g/ml (if not otherwise noted) at 4°C for 24 h. All T cell proliferation assays were performed for 3 days, except for the IL-2-induced proliferations that lasted for 5 days. [³H]Thymidine (1 μ Ci/well) was added for the last 18 h of all assays. The plates were harvested on glass fiber filters and analyzed in a beta scintillation counter (Wallac Oy, Turku, Finland). For NK cell proliferation assays, cells were seeded at 4×10^4 cells/well in U-bottom 96-well plates and cultured in IL-2 at 1,000 U/ml with the indicated Ab. The NK cell proliferation assays were performed for 5 days and measured, as above. In proliferations using CFSE, purified B6 CD8⁺, CD4⁺ T, or DBA/2 CD8⁺ T cells were incubated with 1 μ M CFSE for 10 min at 37°C, washed twice, and cultured at 1.5×10^5 /well with 2×10^5 DX5⁺ IL-2-activated NK cells or irradiated (20,000 rad) P815 cells in a 12-well plate precoated with anti-CD3 Ab. Proliferation was determined by dilution of the CFSE dye by flow cytometry.

Construction of the m2B4-Ig expression vector and production of the 2B4-Ig fusion protein

Total cellular RNA was extracted from DX5⁺ cells using the TRIzol RNA isolation method (Life Technologies, Rockville, MD), followed by cDNA synthesis using the first-strand cDNA synthesis kit (Pharmacia Amersham Biotech, Piscataway, NJ), according to the protocol using pd(N)6 primers.

The cDNA was used as template in a PCR with the primers 2B4-forward (5'-GATCAAGCTTCGACCATGTTGGGGCAAG CTG-3') and 2B4-reverse (5'-GATCGGATCCGCATTCGAAGGGACACTTTGAC-3') containing a restriction site for *Hind*III and *Bam*HI, respectively. The cycling parameters were as follows: 95°C, 3 min; 35 cycles, 95°C, 30 s; 55°C, 45 s; 72°C, 120 s; 72°C, 10 min. The resulting PCR product was digested with *Hind*III and *Bam*HI and cloned into the plasmid pcDNA1.1/Amp-hIg, 5' of a DNA fragment encoding for the Fc part of human IgG1. After verifying the construct by sequencing, vectors containing the m2B4-Ig sequence were transfected into COS-1 cells (32). After 14 days, the culture supernatant was collected and applied to a protein G-Sepharose column. Bound m2B4-Ig molecules were eluted with imidazole and dialyzed against PBS. Purity and integrity were analyzed by silver staining and Western blotting.

CD8⁺ T cell stimulation for CD69 expression

A total of 3×10^5 freshly purified CD8⁺ T cells was cultured in anti-CD3-coated (at concentrations indicated) 48-well plates in 500 μ l of complete α MEM for 3 h alone or in the presence of 3×10^5 irradiated P815 cells. After culture, the cells were stained with anti-CD69 FITC and anti-CD8 α PE and analyzed by flow cytometry.

Statistical analysis

Student's two-sample *t* test was used, assuming unequal variances when comparing triplicates from the different treatments with controls within each experiment, or the paired two-sample *t* test when comparing groups of combined experiments.

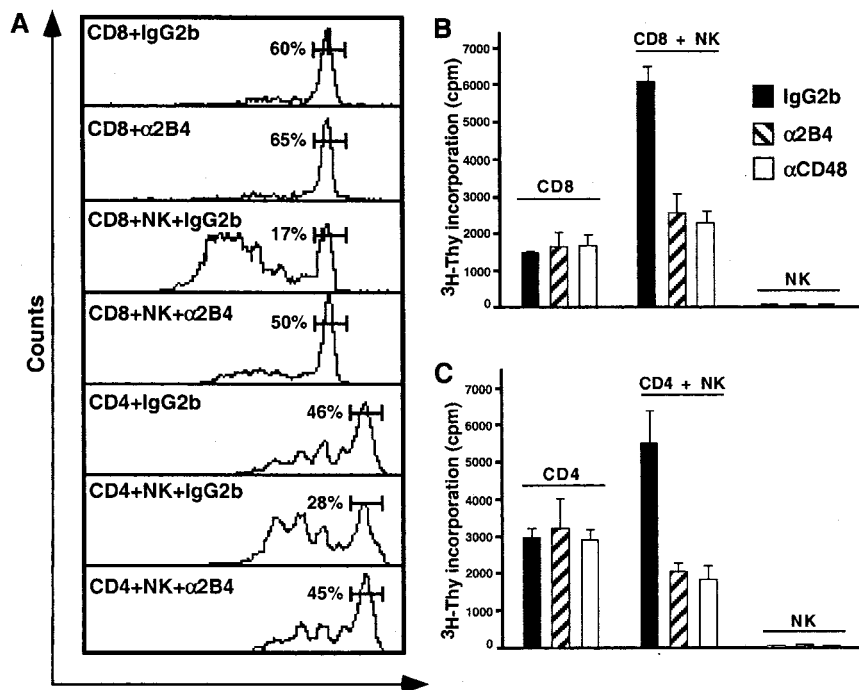
Results

NK cells enhance the anti-CD3-induced proliferation of CD8⁺ and CD4⁺ T cells through 2B4/CD48 interactions

To investigate whether NK cells could directly enhance T cell proliferation, we used a system in which CD8⁺ and CD4⁺ T cells were stimulated with immobilized anti-CD3 Ab in the presence or absence of IL-2-activated NK cells. To visualize the T cell proliferation, CD8⁺ and CD4⁺ T cells were labeled with CFSE, which allows the measurement of proliferating cells by flow cytometry. As shown in Fig. 1A, the addition of NK cells to CFSE-labeled CD8⁺ and CD4⁺ T cells significantly increased T cell proliferation. The addition of anti-2B4 Ab to these cultures reduced the proportion of proliferating CD8⁺ and CD4⁺ T cells to background levels. T cells cultured in the absence of NK cells were not affected by the addition of anti-2B4 Ab (Fig. 1A, *top two histograms*; data not shown). These results suggest that the enhanced proliferation of T cells upon the addition of NK cells was mediated through cell-cell contact. In support of this notion, the NK cell-mediated stimulation of T cell proliferation was abrogated if the NK cells were separated from the T cells by a cell-impermeable porous membrane (data not shown).

NK cell-mediated enhancement of T cell proliferation was also confirmed by measuring [³H]thymidine incorporation. The addition of IL-2-activated NK cells to the T cell cultures clearly enhanced the proliferative response of both CD8⁺ and CD4⁺ T cells (Fig. 1, B and C). Under these conditions, the enhanced proliferation was reversed either by Ab to 2B4 or to its only known ligand CD48, whereas addition of an isotype control Ab or anti-NK1.1 Ab, another activating molecule expressed on NK cells, had no effect (Fig. 1, B and C; data not shown). Additional control experiments revealed that T cell proliferation was not affected by anti-2B4 Ab in the absence of NK cells (Fig. 1, B and C), nor did NK cells proliferate to any major extent when cultured alone (Fig. 1, B and C). However, to exclude that even a minor proliferation of NK cells would give rise to the enhanced thymidine uptake observed when mixed with T cells, NK cells were treated with mitomycin C to inhibit cell division. Mitomycin C-treated NK cells enhanced the T cell proliferation to the same degree as the untreated NK cells (data not shown).

FIGURE 1. NK cell-mediated enhancement of T cell proliferation in an APC-free system. *A*, CFSE-labeled B6 CD8⁺ T cells or CD4⁺ T cells cultured in anti-CD3-coated plates (1 μ g/ml) in the presence or absence of NK cells and Ab. The fluorescence intensity was determined by flow cytometry. Numbers represent percentage of nondividing cells. *B*, CD8⁺ T cells, or *C*, CD4⁺ T cells cultured in anti-CD3-coated plates (1 μ g/ml) in the presence or absence of NK cells and Ab: ■, IgG2b; ▨, α 2B4; □, α CD48. Proliferation was measured by [³H]thymidine incorporation during the last 18 h. Results are expressed as mean cpm \pm SD of triplicate cultures. One representative result of three (*A*) and two (*B* and *C*) independent experiments is shown.



The 2B4/CD48 interactions regulate Ag-driven proliferation of CD8⁺ and CD4⁺ T cells in the presence of NK cells

We next investigated whether 2B4 expression on NK cells could also regulate Ag-specific T cell proliferation. CD8⁺ or CD4⁺ TCR-Tg T cells were cocultured with NK cells and Ag-pulsed DC in the absence or presence of anti-2B4 or anti-CD48 Ab. In these systems, NK cells increased proliferation of T cells, although the degree to which this occurred varied between different experiments. In experiments in which efficient stimulation of T cell proliferation was seen, blocking of either 2B4 or CD48 significantly reduced the proliferative response of both CD8⁺ and CD4⁺ T cells (Fig. 2, *A* and *B*). In contrast, no decrease in proliferation was observed upon addition of an isotype control or anti-NK1.1 Ab (Fig. 2, *A* and *B*). Importantly, proliferation was not affected by anti-2B4 Ab in the absence of NK cells.

NK cells can kill autologous DC *in vitro* in a perforin-dependent manner (33, 34). Because cross-linking of 2B4 is known to enhance NK cell-mediated cytotoxicity, there was a possibility in the present setting that the anti-2B4 Ab triggered NK cells to lyse peptide-loaded DC, which would result in a relatively reduced in-

duction of Ag-specific T cell response (15, 21, 23, 24). Therefore, similar experiments were performed using NK cells from perforin-deficient mice. In addition, anti-Fas ligand (FasL) Ab was added to the cultures to block any possible Fas-FasL-mediated killing of DC. As shown in Fig. 2C, anti-2B4 Ab significantly reduced the CD8⁺ T cell proliferation even in cocultures using perforin-deficient NK cells in combination with anti-FasL Ab. Similar observations were also made using formaldehyde-fixed DC not being susceptible to NK cell-mediated lysis (data not shown). Fab of anti-2B4 Ab were also able to block T cell proliferation in the presence of NK cells, suggesting that the reduced proliferation was not due to cross-linking activation of NK cells by 2B4 cross-linking or Ab-dependent cellular cytotoxicity on the DC (data not shown).

NK cells make up ~5% of all lymphocytes in the peripheral blood and spleen. NK cells are also detectable in lymph nodes, albeit at low numbers. The relatively lower numbers of NK cells than T cells in peripheral blood and secondary lymphoid organs led us to address how many NK cells were required to enhance CD8⁺ T cell proliferation. As few as 1 NK cell per 16 T cells

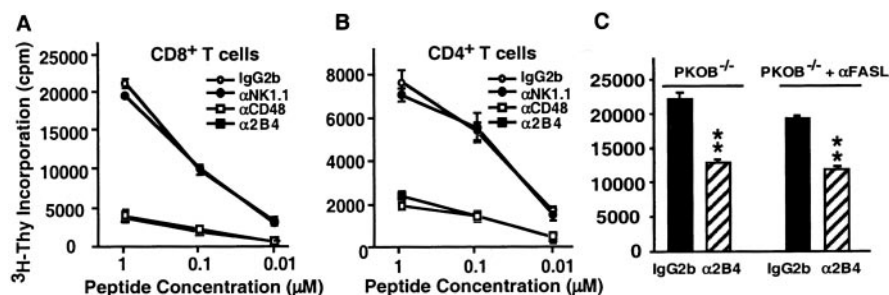


FIGURE 2. The 2B4/CD48 interactions regulate Ag-driven proliferation of CD4⁺ and CD8⁺ T cells in the presence of NK cells. TCR-Tg T cells were cocultured with H-2-syngeneic NK cells (B6 or B10.D2) and DC loaded with specific peptide at concentrations indicated and various Ab, as shown. *A*, F5 TCR-Tg CD8⁺ T cells, or *B*, DO11.10 TCR-Tg CD4⁺ T cells. *C*, F5 TCR-Tg CD8⁺ T cells cocultured with DC loaded with 1 μ M specific peptide and perforin-deficient (PKOB^{-/-}) NK cells in the presence or absence of anti-FasL Ab, and IgG2b or anti-2B4 Ab. Proliferation was measured by [³H]thymidine incorporation. Results are expressed as mean cpm \pm SD of triplicate cultures. **, $p < 0.01$ compared with cultures with IgG2b. One representative result of three (*A* and *B*) or two (*C*) independent experiments is shown.

enhanced the anti-CD3-induced T cell proliferation (Fig. 3). This result suggests that activated NK cells may affect T cell proliferation in organs and tissues in which NK cells are relatively few in numbers.

P815 tumor cells expressing 2B4 enhance the proliferation of CD8⁺ T cells

To directly investigate whether 2B4 expression alone is sufficient to enhance T cell proliferation, we transfected CD48-negative P815 tumor cells with a mutant form of 2B4 that has a truncated intracellular domain, to obviate the signaling potential of the molecule. CFSE-labeled CD8⁺ T cells from DBA/2 mice were cultured in a high dose of IL-2 alone or with irradiated P815-2B4 cells or P815 control cells for 5 days (Fig. 4, A–H). The proliferative response of T cells cultured alone (Fig. 4A) was equivalent to that of T cells cultured with P815 wild type (wt) (Fig. 4C) or with P815-K^b, used as a control for the transfection (Fig. 4H). In contrast, the addition of P815-2B4 cells to the cultures resulted in a marked increase in cell division (Fig. 4E). Notably, the addition of anti-2B4 Ab reversed the proliferation nearly to background levels (Fig. 4F), whereas there was no effect on the proliferation of T cells cultured alone (Fig. 4B) or with P815 wt (Fig. 4D). Similarly, Fab' fragments of the anti-2B4 Ab also reduced the 2B4-mediated increase in T cell proliferation (Fig. 4G).

The 2B4 binds exclusively to CD48

From the present and previously published reports, it could not formally be excluded that 2B4 bound to molecules other than CD48. To address this, we produced a fusion protein composed of the extracellular domain of murine 2B4 and a human IgG1 tail, which was used to stain B6 and CD48^{−/−} splenocytes. All B6 lymphocytes stained positively for 2B4-Ig, whereas no binding was detected on CD48^{−/−} lymphocytes, suggesting that 2B4 binds specifically to CD48 (Fig. 5A). To confirm that the effects of 2B4 were mediated through CD48 on the proliferating T cells, anti-2B4 and anti-CD48 Ab were added together to anti-CD3-stimulated CD8⁺ T cells and NK cells. No additive effect was observed when combining the two Ab, which suggested that the anti-2B4 Ab specifically blocked the interaction between 2B4 and CD48 (Fig. 5B).

When CD4⁺ T cells purified from CD48^{−/−} mice were cocultured with NK cells, no significant increase in T cell proliferation was observed (Fig. 5C). Nor did anti-2B4 or anti-CD48 Ab affect the T cell responses (Fig. 5C). When CD8⁺ T cells purified from

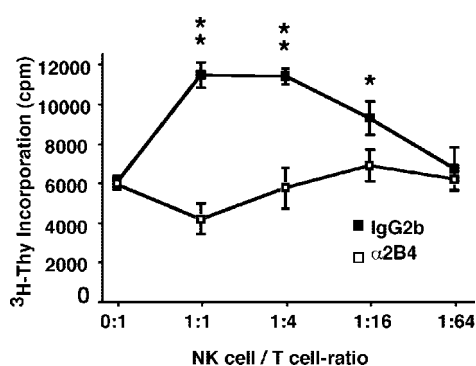


FIGURE 3. Low numbers of NK cells are sufficient to enhance T cell proliferation. B6 CD8⁺ T cells (30,000/well) were cultured in anti-CD3-coated plates with decreasing numbers of NK cells in the presence of Ab. Proliferation was measured by [³H]thymidine incorporation. Results are expressed as mean cpm ± SE of triplicate cultures. **, $p < 0.01$; *, $p < 0.05$ compared with cultures without NK cells. Shown is the result of two independent experiments pooled.

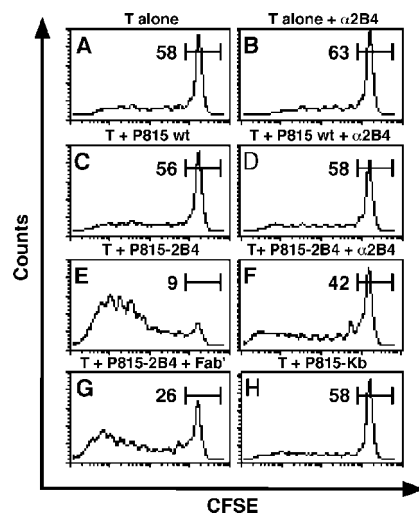


FIGURE 4. The 2B4-expressing P815 tumor cells enhance the proliferation of CD8⁺ T cells. CFSE-labeled DBA/2 CD8⁺ T cells cultured alone or with irradiated P815 tumor cells in the presence of IL-2. Proliferation was determined as decreased fluorescence intensity measured by flow cytometry: A, T cells alone + IgG2b; B, T cells alone + anti-2B4; C, T cells with P815 wt + IgG2b; D, T cells with P815 wt + anti-2B4; E, T cells with P815-2B4 + IgG2b; F, T cells with P815-2B4 + anti-2B4; G, T cells with P815-2B4 + anti-2B4 Fab'; H, T cells with P815-K^b + IgG2b. Numbers represent percentage of nonproliferating cells. One representative result of three independent experiments is shown.

CD48^{−/−} mice were cocultured with wt NK cells, an increase in T cell proliferation was observed (Fig. 5D). The latter increase could for obvious reasons not be due to 2B4-CD48 interactions, nor was the proliferation decreased by the presence of anti-2B4 or anti-CD48 Ab. The reason for this effect of NK cells on CD48-deficient CD8⁺ T cells is not known, and was not subject for further analysis. It is possible, however, that the CD48-deficient CD8⁺ T cells retain some compensatory mechanisms in the absence of CD48-dependent interactions. These results confirm the specificity of the anti-2B4 and anti-CD48 Ab used in our studies.

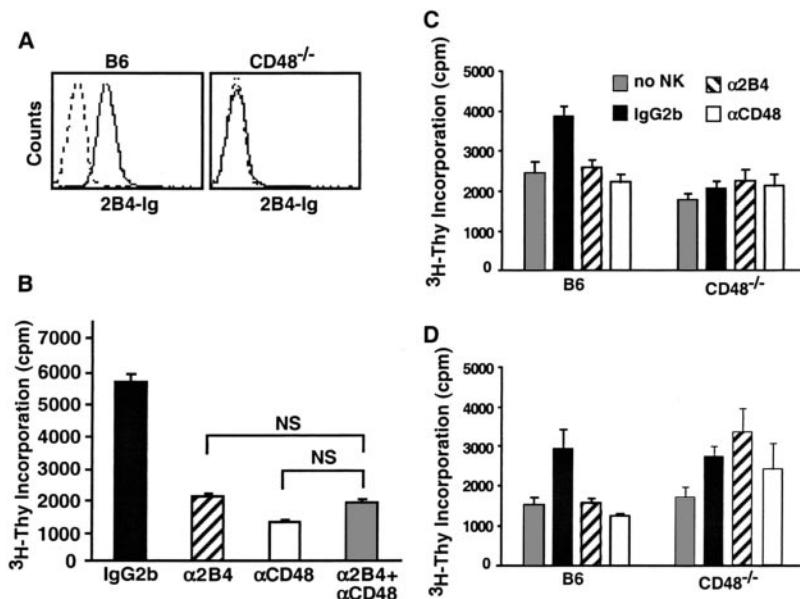
The 2B4-expressing cells enhance the activation of CD8⁺ T cells

The expression of CD69, a marker of recent activation, was examined on anti-CD3-stimulated CD8⁺ T cells that were cocultured with or without 2B4-expressing cells. Strikingly, coculture with P815-2B4 cells increased the fraction of CD69⁺ T cells from ~15 to ~50% as early as 3 h after anti-CD3 stimulation (Fig. 6). The addition of anti-2B4 Ab reduced the number of CD69⁺ T cells to ~20%. In contrast, coculture with P815 wt cells had only a minor effect on the CD69 expression of anti-CD3-stimulated CD8⁺ T cells (Fig. 6). Similarly, coculture with NK cells significantly increased the number of CD69⁺ T cells through 2B4/CD48 interactions (data not shown). Thus, costimulation through 2B4/CD48 interactions not only affected proliferation, but also enhanced short-term activation of T cells.

Increased NK cell proliferation upon 2B4/CD48 interaction

To examine whether NK cell proliferation could also be regulated by 2B4/CD48 interactions, freshly purified murine splenic NK cells were cultured in a high dose of IL-2 (1000 U/ml) for 5 days in the presence of Ab. In these cultures, NK cell proliferation was reduced upon blocking either 2B4 or CD48 (Fig. 7). No additional blocking effect was observed when combining anti-2B4 and anti-CD48 Ab (Fig. 7). Taken together, these results show that NK cells

FIGURE 5. The 2B4 binds no ligand other than CD48. Splenocytes prepared from B6 and CD48^{-/-} mice were stained with 2B4-Ig (solid line) or human Ig (dashed line) and analyzed by flow cytometry. *B*, B6 CD8⁺ cells cultured in anti-CD3-coated plates (1 μ g/ml) with NK cells in the presence of various Ab, as shown. Results are expressed as mean cpm \pm SD of triplicate cultures. *C*, CD4⁺ T cells, or *D*, CD8⁺ T cells from B6 and CD48^{-/-} cultured in anti-CD3-coated plates (1 μ g/ml) in the absence or presence of NK cells in the presence of Ab, as shown: \square , T cells alone + IgG2b; \blacksquare , T cells + NK cells + IgG2b; \square , T cells + NK cells + anti-2B4; \blacksquare , T cells + NK cells + anti-CD48. Shown is the mean [³H]thymidine incorporation \pm SE of two (*C*) or three (*D*) experiments pooled.



augment the proliferation of neighboring T cells as well as other NK cells through direct cell-cell contact via 2B4/CD48 interactions.

Discussion

In the present study, we demonstrate that NK cells enhanced the proliferation of both CD8⁺ and CD4⁺ T cells in response to specific Ag and CD3 cross-linking. Addition of P815 tumor cells transfected with the gene encoding 2B4 (P815-2B4) also enhanced the T cell proliferation in response to IL-2, whereas the addition of P815 wt cells had no effect. The enhancement in proliferation mediated by NK cells or P815-2B4 cells was reversed by anti-2B4 or anti-CD48 Ab. Strikingly, 2B4 displayed immediate effects on T cell activation, because 2B4-expressing cells increased the fraction of CD69-expressing CD8⁺ T cells as early as 3 h after anti-CD3-induced activation. Again, this effect was reversed by the addition of anti-2B4 Ab. These results suggest that the 2B4 molecule acts as a ligand for enhancing activation and proliferation of neighboring T cells through CD48. Furthermore, the IL-2-induced proliferation of both murine and human NK cells was markedly reduced when anti-2B4 or anti-CD48 Ab were added to the cultures (Fig. 7; our unpublished data). These data suggest that 2B4/CD48 interactions play an important role in the proliferation of both T cells and NK cells.

This present study implies that 2B4 bound specifically to CD48, as demonstrated by the inability of a 2B4-Ig fusion protein to bind cells from CD48^{-/-} mice. Furthermore, anti-2B4 Ab had no effect on the proliferation of CD48^{-/-} T cells that were cocultured with NK cells. These results suggest that the anti-2B4 Ab specifically interrupted binding between 2B4 and CD48. The 2B4/CD48 interaction is known to be 5–10 times stronger than the CD2/CD48 interaction.

Interestingly, CD2^{-/-} mice have a similar phenotype to wt mice, whereas T cells from CD48^{-/-} mice have a severe reduction in proliferation and IL-2 production in response to lectins, anti-CD3 Ab, and alloantigens (30, 35–37). Possibly, 2B4 compensates for the lack of CD2 in CD2^{-/-} mice, which may explain why the phenotype of CD2^{-/-} mice is less pronounced. Related to this issue, we have recently shown that memory-like CD8⁺ T cells up-regulate 2B4 upon activation in vitro and in vivo (19). In cultures containing 2B4-expressing T cells, the interaction between

CD2 and CD48 no longer influenced T cell proliferation (19). Therefore, 2B4 may be a more potent ligand than CD2 for co-stimulating lymphocytes through CD48. Based on our present findings, one may predict that 2B4-deficient mice could exhibit reduced T cell responses when additional costimulation is required to drive efficient proliferation of CD4⁺ and CD8⁺ T cells. For

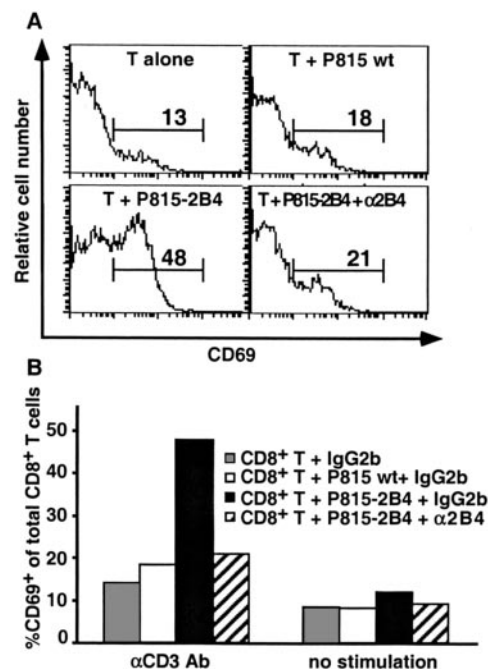


FIGURE 6. The 2B4-expressing cells enhance the activation of CD8⁺ T cells. CD8⁺ T cells were cultured in anti-CD3-coated (0.1 μ g/ml) plates for 3 h alone or in the presence of P815 wt or P815-2B4 cells and Ab, as shown. After culture, the cells were analyzed for CD69 expression (a marker for early activation) when gated on CD8⁺ T cells by flow cytometry. *A*, Histograms showing CD69 expression on CD8⁺ T cells from the cultures. *B*, \square , T cells alone plus IgG2b; \square , T cells plus P815 wt and IgG2b; \blacksquare , T cells plus P815-2B4 and IgG2b; \square , T cells plus P815-2B4 and anti-2B4 Ab. Results are expressed as percentage of CD69⁺ cells of total CD8⁺ T cells. One representative result of two independent experiments is shown.

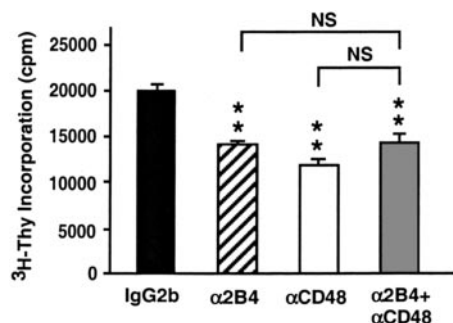


FIGURE 7. NK cell proliferation increases upon 2B4/CD48 interaction. Murine NK cells were stimulated with IL-2 (1000 U/ml) in the presence of Ab, as shown. Proliferation was measured by [³H]thymidine incorporation. Results are expressed as mean cpm \pm SD of triplicate cultures. **, $p < 0.01$ compared with cultures with IgG2b. NS, no significant difference between the two bars. One representative result of three independent experiments is shown.

example, 2B4/CD48 interactions may be important for T cells to mount a sufficient response against an Ag/MHC complex that binds to a given TCR with low affinity. The 2B4^{-/-} mice may also have difficulty in sustaining long-term T cell memory, because 2B4 also enhances cytokine-induced proliferation of memory CD8⁺ T cells. Furthermore, expansion of NK cells may be hampered in the 2B4^{-/-} mice.

The present study demonstrates that, apart from being an inhibitory or activating receptor on NK cells (38, 39), 2B4 can act as a ligand for CD48 expressed on other cells. Cross-linking of CD48 by specific Ab has previously been shown to increase the proliferative response of anti-CD3-stimulated CD8⁺ T cells (40). The present study corroborates the conclusions of that study, by demonstrating that 2B4, a natural ligand for CD48, constitutively expressed on NK cells stimulates proliferation of anti-CD3- as well as specific Ag-stimulated T cells. Although CD48 is a GPI-anchored receptor and lacks intracytoplasmic domains, it can be physically associated to G proteins or to the SRC family of tyrosine kinases in glycolipid-enriched microdomains of the cell membrane (41, 42). This may be mediated by Lck, which is phosphorylated upon CD48 cross-linking (43–45). Interestingly, *lck*^{-/-} CD8⁺ T cells expressed higher levels of CD48 when compared with wt T cells (our unpublished data). Additionally, *lck*^{-/-} NK cells expressed higher levels of 2B4 than wt NK cells. The increased expression level of 2B4 and CD48 may be a compensatory mechanism to achieve efficient cross talk between NK and T cells in the absence of Lck. However, exactly what signals led to the enhancement of proliferation through 2B4/CD48 interactions remains to be determined.

Although cross-linking of 2B4 on NK cells stimulates lytic activity, IFN- γ secretion, and granule exocytosis, the function of 2B4 expressed on T cells is not clear (15, 18, 46). Kumar and colleagues (47) have shown that 2B4/CD48 interactions between T cells can augment CTL activity. These data suggest that ligand interaction among T cells themselves may play a role not only in improving T cell proliferation, but also in enhancing CTL function when these T cells encounter Ags.

T-T cell interactions have recently been described as important players in the generation of T cell memory (25). Briefly, the generation of memory CD8⁺ T cells, with an enhanced capacity for cell division and cytokine secretion, requires CD4 help, but not CD40 expression by the APC. Activated CD4⁺ and CD8⁺ T cells express CD40 and, in the absence of this protein, CD8⁺ T cells are unable to receive CD4 help and to differentiate into memory cells

(25). These results suggest that, like B cells, CD8⁺ T cells receive CD4 help directly through CD40, and that this interaction is fundamental for generation of CD8⁺ T cell memory (25). These studies typify the complex cross talk between different T lymphocyte populations. Our study involving interactions between NK and T cells supports and adds to these previous findings, and furthermore highlights a possible role for cellular interactions between members of the innate and the adaptive immune systems. Our present study also suggests that CD48 can act as a costimulatory receptor and support NK cell proliferation through interaction with other NK cells expressing 2B4. We also found that 2B4 expression is up-regulated on NK cells upon poly(I:C) and IL-2 stimulation (our unpublished data). This suggests that 2B4/CD48 interactions could be important for quickly attaining adequate numbers of activated NK cells at sites of infection.

The localization of NK cells in lymphoid organs has been analyzed in detail (48). It was recently demonstrated that numbers of NK cells within various tissues as well as their specific location changed during murine CMV infection. NK cells were found in close physical proximity to infected cells in both spleen and liver in situ (48, 49). Although speculative at this moment, NK cells recruited to the site of Ag presentation in secondary lymphoid organs or during inflammation could regulate the priming and/or stimulation of adaptive immune cells. In the present study, we demonstrated that low numbers of NK cells can provide help to enhance both CD4⁺ and CD8⁺ T cell responses. However, NK cells could not stimulate T cell proliferation by themselves, but did so in combination with CD3 cross-linking, cytokines, or specific Ag presented by DC. In a physiological setting, one could envisage a menage à trois between mature DC, T cells, and NK cells, in which T cells receive signals 1 and 2 from the DC and additional signals from the NK cells. Similar models were previously proposed for B cell class switching (50). Signals given by DC (CD40-CD40L interaction) and cytokines produced by T cells primed on the DC act synergistically to stimulate class switching in the B cells.

A number of reports have convincingly demonstrated that the cytokine milieu formed by the NK cells early during an infection plays a central role in influencing the outcome of the subsequent humoral and cellular immune responses (4, 6, 8, 9, 51, 52). In addition to this notion, we have demonstrated that direct physical interactions between NK and T cells result in enhanced proliferative responses of the latter.

Acknowledgments

We thank Drs. A. Sharpe and Y. Latchman for generously providing us with CD48^{-/-} splenocytes, Dr. D. Kioussis for the TCR-Tg (F5 Tg) mice, Dr. L. Berg for assistance with technical procedures, and members of the Ljunggren and the Jensen groups for helpful discussions.

References

1. Biron, C. A., and R. M. Welsh. 1982. Activation and role of natural killer cells in virus infections. *Med. Microbiol. Immunol.* 170:155.
2. Tay, C. H., E. Szomolanyi-Tsuda, and R. M. Welsh. 1998. Control of infections by NK cells. *Curr. Top. Microbiol. Immunol.* 230:193.
3. Biron, C. A., and L. Brossay. 2001. NK cells and NKT cells in innate defense against viral infections. *Curr. Opin. Immunol.* 13:458.
4. Bancroft, G. J. 1993. The role of natural killer cells in innate resistance to infection. *Curr. Opin. Immunol.* 5:503.
5. Cerwenka, A., and L. L. Lanier. 2001. Natural killer cells, viruses and cancer. *Nat. Rev. Immunol.* 1:41.
6. Perussia, B. 1991. Lymphokine-activated killer cells, natural killer cells and cytokines. *Curr. Opin. Immunol.* 3:49.
7. Shi, F., H. G. Ljunggren, and N. Sarvetnick. 2001. Innate immunity and autoimmunity: from self-protection to self-destruction. *Trends Immunol.* 22:97.
8. Gray, J. D., and D. A. Horvitz. 1995. Activated human NK cells can stimulate resting B cells to secrete immunoglobulin. *J. Immunol.* 154:5656.
9. Wilder, J. A., C. Y. Koh, and D. Yuan. 1996. The role of NK cells during in vivo antigen-specific antibody responses. *J. Immunol.* 156:146.

10. Tangye, S. G., J. H. Phillips, and L. L. Lanier. 2000. The CD2-subset of the Ig superfamily of cell surface molecules: receptor-ligand pairs expressed by NK cells and other immune cells. *Semin. Immunol.* 12:149.
11. Bachmann, M. F., M. Barner, and M. Kopf. 1999. CD2 sets quantitative thresholds in T cell activation. *J. Exp. Med.* 190:1383.
12. Sido, B., T. J. Dengler, G. Otto, R. Zimmermann, P. Muller, and S. C. Meuer. 1998. Differential immunosuppressive activity of monoclonal CD2 antibodies on allograft rejection versus specific antibody production. *Eur. J. Immunol.* 28:1347.
13. Blazar, B. R., P. A. Taylor, A. Panoskaltis-Mortari, H. Yagita, J. S. Bromberg, and D. A. Vallera. 1998. A critical role for CD48 antigen in regulating alloengraftment and lymphohematopoietic recovery after bone marrow transplantation. *Blood* 92:4453.
14. Punch, J. D., J. Lin, J. Bluestone, and J. S. Bromberg. 1999. CD2 and CD3 receptor-mediated tolerance: constraints on T cell activation. *Transplantation* 67:741.
15. Nakajima, H., M. Cella, H. Langen, A. Friedlein, and M. Colonna. 1999. Activating interactions in human NK cell recognition: the role of 2B4⁺ CD48. *Eur. J. Immunol.* 29:1676.
16. Schuhmachers, G., K. Ariizumi, P. A. Mathew, M. Bennett, V. Kumar, and A. Takashima. 1995. 2B4, a new member of the immunoglobulin gene superfamily, is expressed on murine dendritic epidermal T cells and plays a functional role in their killing of skin tumors. *J. Invest. Dermatol.* 105:592.
17. Colonna, M., H. Nakajima, and M. Cella. 1999. Inhibitory and activating receptors involved in immune surveillance by human NK and myeloid cells. *J. Leukocyte Biol.* 66:718.
18. Valiante, N. M., and G. Trinchieri. 1993. Identification of a novel signal transduction surface molecule on human cytotoxic lymphocytes. *J. Exp. Med.* 178:1397.
19. Kambayashi, T., E. Assarsson, B. J. Chambers, and H. G. Ljunggren. 2001. Cutting edge: regulation of CD8⁺ T cell proliferation by 2B4/CD48 interactions. *J. Immunol.* 167:6706.
20. Brown, M. H., K. Boles, P. A. van der Merwe, V. Kumar, P. A. Mathew, and A. N. Barclay. 1998. 2B4, the natural killer and T cell immunoglobulin superfamily surface protein, is a ligand for CD48. *J. Exp. Med.* 188:2083.
21. Nakajima, H., and M. Colonna. 2000. 2B4: an NK cell activating receptor with unique specificity and signal transduction mechanism. *Hum. Immunol.* 61:39.
22. Chuang, S. S., Y. Lee, S. E. Stepp, P. R. Kumaresan, and P. A. Mathew. 1999. Molecular cloning and characterization of the promoter region of murine natural killer cell receptor 2B4. *Biochim. Biophys. Acta* 1447:244.
23. Kubin, M. Z., D. L. Parshley, W. Din, J. Y. Waugh, T. Davis-Smith, C. A. Smith, B. M. Macduff, R. J. Armitage, W. Chin, L. Cassiano, et al. 1999. Molecular cloning and biological characterization of NK cell activation-inducing ligand, a counterstructure for CD48. *Eur. J. Immunol.* 29:3466.
24. Garni-Wagner, B. A., A. Purohit, P. A. Mathew, M. Bennett, and V. Kumar. 1993. A novel function-associated molecule related to non-MHC-restricted cytotoxicity mediated by activated natural killer cells and T cells. *J. Immunol.* 151:60.
25. Bourgeois, C., B. Rocha, and C. Tanchot. 2002. A role for CD40 expression on CD8⁺ T cells in the generation of CD8⁺ T cell memory. *Science* 297:2060.
26. Mamalaki, C., T. Norton, Y. Tanaka, A. R. Townsend, P. Chandler, E. Simpson, and D. Kioussis. 1992. Thymic depletion and peripheral activation of class I major histocompatibility complex-restricted T cells by soluble peptide in T-cell receptor transgenic mice. *Proc. Natl. Acad. Sci. USA* 89:11342.
27. Murphy, K. M., A. B. Heimberger, and D. Y. Loh. 1990. Induction by antigen of intrathymic apoptosis of CD4⁺CD8⁺TCR^{lo} thymocytes in vivo. *Science* 250:1720.
28. Mombaerts, P., J. Iacomini, R. S. Johnson, K. Herrup, S. Tonegawa, and V. E. Papaioannou. 1992. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* 68:869.
29. Kagi, D., F. Vignaux, B. Ledermann, K. Burki, V. Depraetere, S. Nagata, H. Hengartner, and P. Golstein. 1994. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science* 265:528.
30. Gonzalez-Cabrero, J., C. J. Wise, Y. Latchman, G. J. Freeman, A. H. Sharpe, and H. Reiser. 1999. CD48-deficient mice have a pronounced defect in CD4⁺ T cell activation. *Proc. Natl. Acad. Sci. USA* 96:1019.
31. Inaba, K., R. M. Steinman, M. W. Pack, H. Aya, M. Inaba, T. Sudo, S. Wolpe, and G. Schuler. 1992. Identification of proliferating dendritic cell precursors in mouse blood. *J. Exp. Med.* 175:1157.
32. Ishiura, M., H. Ohashi, T. Uchida, and Y. Okada. 1989. Phage particle-mediated gene transfer of recombinant cosmid to cultured mammalian cells. *Gene* 82:281.
33. Chambers, B. J., M. Salcedo, and H. G. Ljunggren. 1996. Triggering of natural killer cells by the costimulatory molecule CD80 (B7-1). *Immunity* 5:311.
34. Wilson, J. L., L. C. Heffler, J. Charo, A. Scheynius, M. T. Bejarano, and H. G. Ljunggren. 1999. Targeting of human dendritic cells by autologous NK cells. *J. Immunol.* 163:6365.
35. Evans, C. F., G. F. Rall, N. Killeen, D. Littman, and M. B. Oldstone. 1993. CD2-deficient mice generate virus-specific cytotoxic T lymphocytes upon infection with lymphocytic choriomeningitis virus. *J. Immunol.* 151:6259.
36. Davis, S. J., and P. A. van der Merwe. 1996. The structure and ligand interactions of CD2: implications for T-cell function. *Immunol. Today* 17:177.
37. Cabrero, J. G., G. J. Freeman, and H. Reiser. 1998. The murine Cd48 gene: allelic polymorphism in the IgV-like region. *Eur. J. Immunogenet.* 25:421.
38. Schatzle, J. D., S. Sheu, S. E. Stepp, P. A. Mathew, M. Bennett, and V. Kumar. 1999. Characterization of inhibitory and stimulatory forms of the murine natural killer cell receptor 2B4. *Proc. Natl. Acad. Sci. USA* 96:3870.
39. Stepp, S. E., J. D. Schatzle, M. Bennett, V. Kumar, and P. A. Mathew. 1999. Gene structure of the murine NK cell receptor 2B4: presence of two alternatively spliced isoforms with distinct cytoplasmic domains. *Eur. J. Immunol.* 29:2392.
40. Kato, K., M. Koyanagi, H. Okada, T. Takanashi, Y. W. Wong, A. F. Williams, K. Okumura, and H. Yagita. 1992. CD48 is a counter-receptor for mouse CD2 and is involved in T cell activation. *J. Exp. Med.* 176:1241.
41. Solomon, K. R., C. E. Rudd, and R. W. Finberg. 1996. The association between glycosylphosphatidylinositol-anchored proteins and heterotrimeric G protein α subunits in lymphocytes. *Proc. Natl. Acad. Sci. USA* 93:6053.
42. Cebecauer, M., J. Cerny, and V. Horejsi. 1998. Incorporation of leukocyte GPI-anchored proteins and protein tyrosine kinases into lipid-rich membrane domains of COS-7 cells. *Biochem. Biophys. Res. Commun.* 243:706.
43. Loertscher, R., and P. Lavery. 2002. The role of glycosyl phosphatidyl inositol (GPI)-anchored cell surface proteins in T-cell activation. *Transplant Immunol.* 9:93.
44. Garnett, D., A. N. Barclay, A. M. Carmo, and A. D. Beyers. 1993. The association of the protein tyrosine kinases p56^{lck} and p60^{src} with the glycosyl phosphatidylinositol-anchored proteins Thy-1 and CD48 in rat thymocytes is dependent on the state of cellular activation. *Eur. J. Immunol.* 23:2540.
45. Patel, V. P., M. Moran, T. A. Low, and M. C. Miceli. 2001. A molecular framework for two-step T cell signaling: Lck Src homology 3 mutations discriminate distinctly regulated lipid raft reorganization events. *J. Immunol.* 166:754.
46. Boles, K. S., H. Nakajima, M. Colonna, S. S. Chuang, S. E. Stepp, M. Bennett, V. Kumar, and P. A. Mathew. 1999. Molecular characterization of a novel human natural killer cell receptor homologous to mouse 2B4. *Tissue Antigens* 54:27.
47. Lee, K. M., S. Bhawan, T. Majima, H. Wei, M. I. Nishimura, H. Yagita, and V. Kumar. 2003. Cutting edge: the NK cell receptor 2B4 augments antigen-specific T cell cytotoxicity through CD48 ligation on neighboring T cells. *J. Immunol.* 170:4881.
48. Brown, M. G., A. O. Dokun, J. W. Heusel, H. R. Smith, D. L. Beckman, E. A. Blattenberger, C. E. Dubbelde, L. R. Stone, A. A. Scalzo, and W. M. Yokoyama. 2001. Vital involvement of a natural killer cell activation receptor in resistance to viral infection. *Science* 292:934.
49. Dokun, A. O., S. Kim, H. R. Smith, H. S. Kang, D. T. Chu, and W. M. Yokoyama. 2001. Specific and nonspecific NK cell activation during virus infection. *Nat. Immunol.* 2:951.
50. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392:245.
51. Kos, F. J. 1998. Regulation of adaptive immunity by natural killer cells. *Immunol. Res.* 17:303.
52. Kos, F. J., and E. G. Engleman. 1996. Role of natural killer cells in the generation of influenza virus-specific cytotoxic T cells. *Cell. Immunol.* 173:1.