

UCSF

UC San Francisco Previously Published Works

Title

Functional expression of B7/BB1 on activated T lymphocytes.

Permalink

<https://escholarship.org/uc/item/6pw7t0cq>

Journal

The Journal of experimental medicine, 177(3)

ISSN

0022-1007

Authors

Azuma, M
Yssel, H
Phillips, JH
[et al.](#)

Publication Date

1993-03-01

DOI

10.1084/jem.177.3.845

Peer reviewed

Functional Expression of B7/BB1 on Activated T Lymphocytes

By Miyuki Azuma,¹ Hans Yssel,¹ Joseph H. Phillips, Hergen Spits, and Lewis L. Lanier

From the DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, California 94304

Summary

B7/BB1 is a membrane differentiation antigen expressed on activated B cells, macrophages, and dendritic cells that binds to a counter-receptor, CD28, expressed on T lymphocytes and thymocytes. Interaction between CD28 and B7 results in potent costimulation of T cell activation initiated via the CD3/T cell receptor complex. We now report that B7 is also expressed on activated human peripheral blood T cells, CD4 T cell clones, CD8 T cell clones, and natural killer cell clones. B7 appears relatively late after T cell activation, can be detected on both CD4 and CD8 T cell subsets, and is present on antigen-specific, major histocompatibility complex-restricted CD4 and CD8 T cell clones. Expression of B7 on activated T cells was confirmed by immunoprecipitation from ¹²⁵I-labeled activated T cells and by detection of B7 transcripts. A B7⁺ CD4⁺ T cell clone was able to stimulate a primary allogeneic mixed lymphocyte response using small, resting peripheral blood T cells as responders. The alloantigen-induced proliferative response and cytokine production was partially inhibited by anti-B7 monoclonal antibody. Since activated T cells can coexpress both CD28 and its counter-receptor, B7, this suggests that activated T cells may be capable of autocrine costimulation via the CD28 activation pathway.

B7/BB1 is a cell surface antigen expressed on activated B cells, macrophages, and dendritic cells (1–3). While B7 is constitutively expressed on dendritic cells (3), it is only present in substantial levels after activation of B cells and monocytes. This antigen is upregulated on B cells after infection with EBV (2) or after stimulation with anti-Ig (1) or anti-HLA-DR (4). Levels of B7 expression induced by anti-Ig can be increased by IL-2 or IL-4 (5). IFN- γ is capable of inducing B7 expression on monocytes (6). B7 is a 50–70-kD glycoprotein and is encoded by a gene that is a member of the Ig superfamily (7).

B7 binds to CD28 (8), a cell surface disulfide-linked homodimer that is constitutively expressed on thymocytes and the majority of T lymphocytes (9, 10). The interaction between CD28 and B7 results in augmentation of T cell activation initiated through the TCR (4, 8, 11–13). CD28 costimulation of T cells results in induction of IL-2 transcription (14) and stabilization of cytokine mRNA (15), and is resistant to inhibition by cyclosporine A (16). In a prior study, it was observed that HTLV-I-transformed T cells may also express B7 (17), suggesting the possibility that T cells may coexpress both CD28 and its counter-receptor under certain

circumstances. Herein, we have investigated whether normal, nontransformed T cells are capable of expressing functional B7 antigens.

Materials and Methods

Preparation of Lymphocytes and Cell Culture. Human peripheral blood was obtained from the Stanford Blood Center (Palo Alto, CA). PBMC were isolated by Ficoll/Hypaque gradient centrifugation. Small, resting T cells were isolated using Percoll density gradients after removal of B cells and monocytes by plastic and nylon wool adherence, as previously described (13). Cells were cultured in Yssel's modified Iscove's medium (18) or RPMI 1640 (JRH Biosciences, Lenexa, KS) containing 10% FCS (JRH Biosciences), L-glutamine, and antibiotics. Human rIL-2 was generously provided by Dr. Gerard Zurawski (DNAX). A series of CD4⁺ T cell clones, CD8⁺ T cell clones, and NK cell clones was established from normal PBL. Clones were cultured in medium supplemented with rIL-2 and were restimulated using PHA, irradiated PBMC, and irradiated EBV B lymphoblastoid cells, as described previously (18). The properties of the antigen-specific T cell clones have been described previously (19–22).

mAbs, Immunofluorescence, and Flow Cytometry. Methods of immunofluorescent staining, flow cytometry, and data analysis have been described previously (23). Flow cytometry was performed using a FACScan[®] or FACStar Plus[®] (Becton Dickinson & Co., Mountain View, CA). All mAbs were generously provided by Becton Dickinson & Co., unless otherwise noted. BB1 mAb (2) was the

¹ The first two authors contributed equally to these studies.

generous gift of Dr. Ed Clark (University of Washington, Seattle, WA). Anti-B7 mAbs L307 (IgG1) and L308 (IgM) were generated by immunizing C3H/HeJ mice with B7-transfected L cells and fusing immune splenocytes with Sp2/0 myeloma cells, as described previously (24).

Biochemistry. Radioiodination, immunoprecipitation, and electrophoresis were performed as previously described (25, 26). Radioactive proteins were detected using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

Results and Discussion

Expression of B7 on Activated T Lymphocytes and T Cell Clones. Analysis of a series of human T cell clones indicated that the majority expressed B7 on the cell surface, as determined by immunofluorescence and flow cytometry. Expression of B7 on T cell clones was not restricted to a particular subset since both CD3⁺CD4⁺ T cell clones (14 of 16 examined) and CD3⁺CD8⁺ T cell clones (14 of 14 examined) were found. Moreover, B7 was also present on CD3⁻CD56⁺ NK cell clones (five of five examined), but was not detected on freshly isolated PBL NK cells (not shown). The levels of B7 varied between different clones and ranged from very high levels of expression to undetectable. Most of the B7⁺ T cell clones examined coexpressed the counter-receptor, CD28, whereas all B7⁺ NK cell clones were CD28 negative. Representative T and NK cell clones are shown in Fig. 1. Although the antigenic specificity of most of the T cell clones surveyed in our panel was not determined, B7 was detected on several well-characterized T cell clones of known specificity and function. For example, HY-827 is a CD4⁺, HLA-DR3-restricted T cell clone that recognizes a peptide of tetanus toxoid (19), HY-06 is a CD4⁺, HLA-DR3-restricted T cell clone that recognizes mycobacterial HSP-65 antigen (20), AG150 is a CD4⁺, HLA-DR4-restricted T cell clone recognizing the *Der p1* house dust mite allergen (21), and CTL Q66.40 is a CD8⁺, HLA-A2-restricted T cell clone reacting with an influenza peptide (22) (Fig. 1). The presence of B7 on T cell clones was relatively stable over time, although differences in levels of expression were observed.

The presence of B7 on most T cell clones prompted us to examine induction of this antigen on PBL T cells. Consistent with prior findings, B7 was not detected on freshly isolated PBL T cells (Fig. 2). PBMC were stimulated with immobilized anti-CD3, and 100 IU/ml rIL-2 was added to the culture after 5 d. Cells were examined on days 1, 3, 5, 10, and 12 for induction of B7. Using these conditions, B7 appeared gradually on the T cells over the course of culture, and maximal expression occurred after 10 d when >80% of T cells coexpressed B7 (Fig. 2). The majority of both CD4 and CD8 T cells were B7⁺. Consistent results were obtained using PBMC from three independent donors. The relatively late induction and the low levels of expression on some T cell clones likely accounts for prior reports that activated T cells failed to express B7 (2, 17).

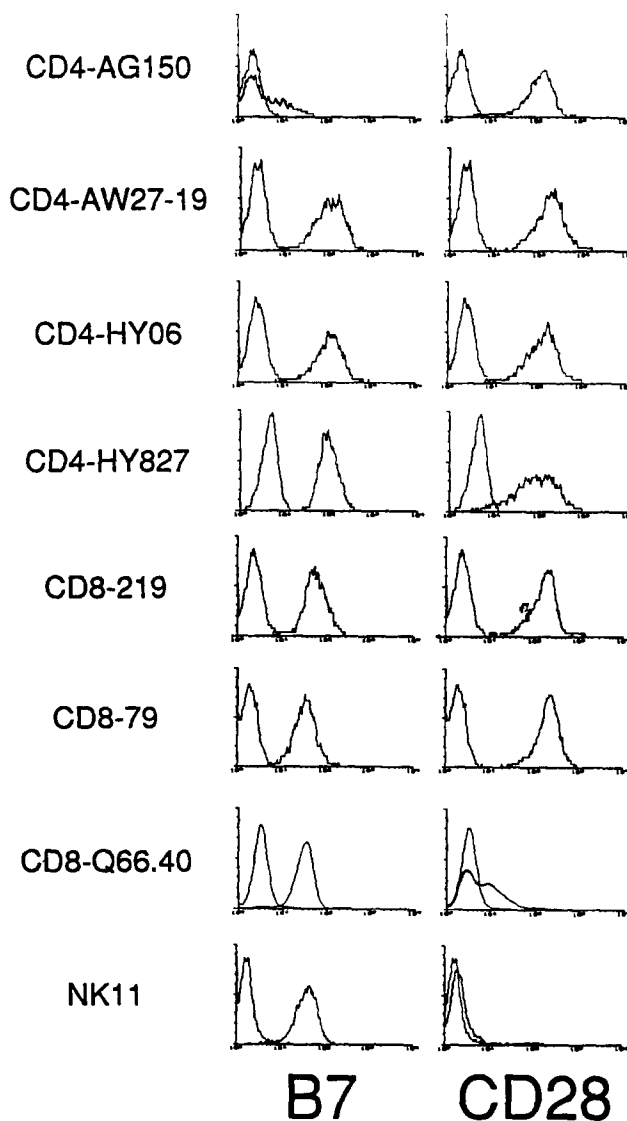


Figure 1. Expression of B7 on T and NK cell clones. Representative CD4⁺ and CD8⁺ T cell clones and a CD3⁻CD56⁺ NK cell clone were stained with PE-conjugated anti-B7 mAb L307, PE-conjugated anti-CD28 mAb L293, or PE-conjugated IgG1 control mAb. Samples were analyzed by flow cytometry. The x-axis represents fluorescence (four-decade log scale) and the y axis represents the relative cell number. Histograms from cells stained with control mAb (nearest the y-axis) are superimposed over the histograms of cells stained with anti-B7 or anti-CD28, as indicated.

Biochemical Analysis of T Cell-associated B7 Antigen. The presence of B7 on activated T cell populations and T cell clones was confirmed by immunofluorescent staining using three different mAbs against B7 (i.e., BB1, L307, L308) (not shown). Specificity of the immunofluorescent staining was substantiated by biochemical verification (Fig. 3). A CD4⁺ T clone (AW27-19) and a polyclonal population of anti-CD3 and rIL-2-activated PBL T cells were ¹²⁵I labeled, detergent lysed, and B7 antigen was immunoprecipitated and analyzed by SDS-

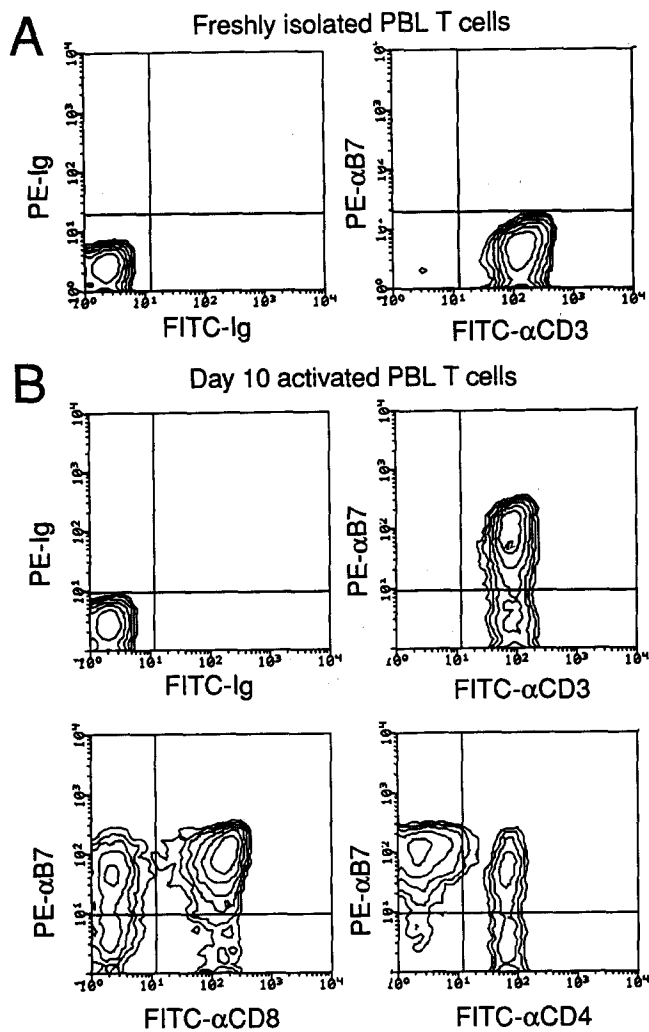


Figure 2. Induction of B7 on PBL T lymphocytes. Plastic tissue culture flasks (Falcon Labware, Lincoln Park, NJ) were coated with 10 $\mu\text{g}/\text{ml}$ anti-CD3 (Leu-4) in PBS for 1 h and then washed extensively. PBMC (10^6 cells/ml) were activated by culture in anti-CD3-coated tissue culture flasks. After 5 d, the media were supplemented with 100 IU/ml rIL-2, and culture was continued. Freshly isolated PBL T cells (A) and day 10 activated PBL T cells (B) were stained with PE-conjugated anti-B7- and FITC-conjugated anti-CD3 (Leu-4), anti-CD4 (Leu-3a), or anti-CD8 α (Leu-2a), or with FITC- and PE-conjugated control Ig. Samples were analyzed by flow cytometry. Data are displayed as contour plots of correlated FITC and PE fluorescence (four-decade log scales). Quadrant markers were positioned to include >98% of control Ig stained cells in the lower left.

PAGE. Similar to the molecular mass of B7 expressed on EBV-transformed B cell lines (1, 2), the B7 glycoprotein on activated T cells was also $\sim 50\text{--}70$ kD. Expression of B7 in NK and T cell clones was further supported by detection of B7 transcripts using reverse transcription and PCR amplification (not shown).

B7⁺ T Cell Clones Stimulate a Primary Alloantigen MLR. Prior studies have established that B7 costimulation is required

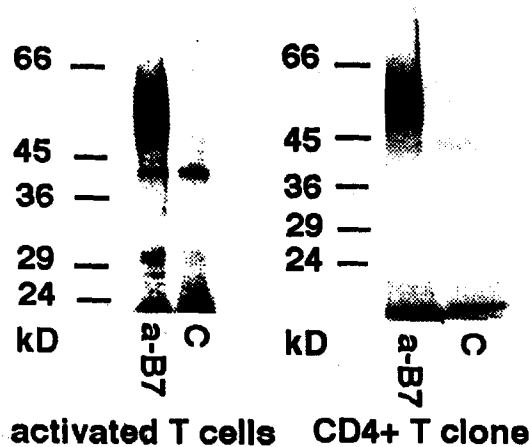


Figure 3. Immunoprecipitation of B7. B7⁺ CD4⁺ T cell clone AW27-19 (see Fig. 1) and a polyclonal population of PBL T cells activated with anti-CD3 and 100 IU/ml rIL-2 for 12 d were labeled with ^{125}I , lysed in 1% NP-40 lysis buffer, and immunoprecipitated with formalin-fixed *Staphylococcus aureus* coated with rabbit anti-mouse Ig and control mouse Ig (C) or anti-B7 mAb L307 (25, 26). Antigens were eluted in sample buffer containing 10% 2-ME and were analyzed using 9% (left) or 10% (right) SDS-PAGE gels.

for the efficient generation of a primary MLR directed against alloantigen (13, 27). mAbs against B7 partially inhibit an alloantigen-induced MLR (3, 4), and transfection of B7-negative B lymphoma cell lines with B7 cDNA substantially augments their ability to stimulate a primary T cell immune response (13, 27). The detection of B7 on activated T cells suggested that this may enable these T cells to act themselves as costimulators of a primary T cell response. Since human activated T cells express both MHC class II and class I antigens and B7, they might be expected to serve as stimulators of an allogeneic MLR. To examine this possibility, a CD4⁺ T cell clone expressing B7 (Fig. 1, CD4-AW27-19) was irradiated and used as the stimulator in a primary MLR culture using freshly isolated small, resting PBL T cells as responders. As shown in Table 1, a vigorous MLR response was observed using small, resting PBL responder T cells from three different donors. Both the alloantigen-induced proliferative response, as well as the secretion of IFN- γ , were partially ($\sim 30\text{--}50\%$) inhibited by the presence of anti-B7 mAb, similar to the level of inhibition observed with anti-CD4 or anti-HLA-DR mAbs (Table 1). These results are comparable to prior reports that anti-B7 is capable of partially inhibiting alloantigen MLR generated with EBV B lymphoblastoid cells as stimulators (3, 4).

Conclusions. B7 is not restricted in expression to "conventional" APC since this glycoprotein is also present on most activated T and NK cells. The ability of B7⁺ activated T cell clones to stimulate a primary allogeneic MLR response substantiates the fact that expression of B7 is functionally competent. Prior studies have demonstrated that activated T and NK cell clones possess the ability to present peptide

Table 1. B7⁺ T Cell Clone AW27-19 Stimulates Primary Alloantigen-induced Proliferative and Cytokine Responses Using Small, Resting T Cells as Responders

Small, resting T responders	Irradiated B7 ⁺ T stimulators	mAb added	[³ H]TdR incorporation		
			Exp. 1	Exp. 2	Exp. 3
				<i>cpm × 10⁻³</i>	
+	+	Control	20.3 (-)*	15.3 (-)	14.7 (-)
+	+	αB7	9.8 (51.7)	9.5 (37.9)	6.3 (57.3)
+	+	αCD4	8.5 (58.1)	7.5 (49.6)	7.7 (45.9)
+	+	αHLA-DR	5.5 (72.4)	7.7 (50.9)	7.4 (49.8)
+	+	αCD4 + HLA-DR	2.5 (87.7)	7.3 (52.3)	2.6 (81.6)
+	-	None	1.0	2.3	1.0
-	+	None	1.5	1.6	2.2
			IFN-γ production		
			Exp. 1	Exp. 2	Exp. 3
				<i>pg/ml</i>	
+	+	Control	5,650 (-)*	6,685 (-)	3,600 (-)
+	+	αB7	2,840 (48.2)	4,750 (28.9)	1,580 (56.1)
+	+	αCD4	2,505 (55.6)	4,200 (37.1)	2,125 (40.9)
+	+	αHLA-DR	1,520 (73.1)	5,915 (11.5)	1,600 (55.5)
+	+	αCD4 + HLA-DR	975 (82.7)	500 (92.5)	870 (75.8)
+	-	None	<50	<50	<50
-	+	None	<50	<50	<50

Freshly isolated small, resting PBL T cells (2×10^5 cells/well) from three different blood donors (Exps. 1-3) were cocultured with irradiated (4,000 rad) B7⁺ T cell clone AW27-19 (2×10^5 cells/well) in 96-well flat-bottomed plates (Falcon Labware) in a total volume of 200 μ l, as indicated. Anti-B7, anti-HLA-DR, and anti-CD4 mAbs were added at 5 μ g/ml. Cultures were incubated at 37°C with 5% CO₂ in a humidified atmosphere for 6 d. Cultures were labeled for the final 4 h with 1 μ Ci/well [³H]thymidine (New England Nuclear, Boston, MA), were harvested using a 96-well plate harvester (LKB, Pharmacia, Piscataway, NJ), and radioactivity was measured using a β scintillation counter. Small, resting PBL T cells isolated by Percoll gradient centrifugation are unable to respond to anti-CD3 stimulation in the absence of appropriate accessory cells (13). The irradiated AW27-19 B7⁺ CD4⁺ T cells used as stimulators were >99% CD3⁺ and did not contain any detectable contaminating B cells or monocytes in the population, as determined using flow cytometric analysis. For determination of IFN- γ secretion, supernatants from MLR cultures were harvested after 48 h, and IFN- γ was measured by an ELISA using anti-IFN- γ -specific mAb (30). Data are expressed as IFN- γ (pg/ml) secreted per 10⁶ cells (lower limit of detection was 50 pg/ml).

* Numbers in parentheses represent percent inhibition, relative to control values.

antigens in an antigen-specific, MHC-restricted fashion, although the antigen-processing capacity of T and NK cells are limited relative to macrophages (28, 29). Since activated T cells can coexpress both CD28 and its counter-receptor,

B7, this suggests the possibility that activated T cells may be capable of autocrine costimulation via the CD28 activation pathway under certain circumstances.

We thank Eleni Callas, Dixie Polakoff, and Jim Cupp for expert assistance with the flow cytometry; Mr. Chiwen Chang for PCR analysis; Dr. Rene de Waal Malefyt for providing T cell clones; and Mr. Bruce Bennett for IFN- γ determination.

The DNAX Research Institute of Molecular and Cellular Biology is supported by Schering Plough Corporation.

Address correspondence to Lewis L. Lanier, Department of Immunology, DNAX Research Institute of Molecular and Cellular Biology, 901 California Avenue, Palo Alto, CA 94304. Miyuki Azuma's present

address is the 2nd Department of Oral and Maxillo-facial Surgery, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113, Japan.

Received for publication 9 November 1992 and in revised form 1 December 1992.

Note added in proof: Sansom and Hall (31) have also recently observed expression of B7 on activated T cells.

References

1. Freedman, A.S., G. Freeman, J.C. Horowitz, J. Daley, and L.M. Nadler. 1987. B7, a B cell-restricted antigen that identifies preactivated B cells. *J. Immunol.* 139:3260.
2. Yokochi, T., R.D. Holly, and E.A. Clark. 1982. B lymphoblast antigen (BB-1) expressed on Epstein-Barr virus-activated B cell blasts, B lymphoblastoid cell lines, and Burkitt's lymphomas. *J. Immunol.* 128:823.
3. Young, J.W., L. Koulova, S.A. Soergel, E.A. Clark, R.M. Steinman, and B. Dupont. 1992. The B7/BB1 antigen provides one of several costimulatory signals for the activation of CD4⁺ T lymphocytes by human blood dendritic cells in vitro. *J. Clin. Immunol.* 90:229.
4. Koulova, L., E.A. Clark, G. Shu, and B. Dupont. 1991. The CD28 ligand B7/BB1 provides costimulatory signal for alloactivation of CD4⁺ T cells. *J. Exp. Med.* 173:759.
5. Valle, A., J.-P. Aubry, I. Durand, and J. Banchemereau. 1991. IL-4 and IL-2 upregulate the expression of antigen B7, the B cell counterstructure to T cell CD28: an amplification mechanism for T-B cell interactions. *Int. Immunol.* 3:229.
6. Freedman, A.S., G.J. Freeman, K. Rhyhart, and L.M. Nadler. 1991. Selective induction of B7/BB-1 on interferon-gamma stimulated monocytes: a potential mechanism for amplification of T-cell activation through the CD28 pathway. *Cell. Immunol.* 137:429.
7. Freeman, G.J., A.S. Freedman, J.M. Segil, G. Lee, J.F. Whitman, and L.M. Nadler. 1989. B7, a new member of the Ig superfamily with unique expression on activated and neoplastic B cells. *J. Immunol.* 143:2714.
8. Linsley, P.S., E.A. Clark, and J.A. Ledbetter. 1990. The T cell antigen, CD28, mediates adhesion with B cells by interacting with the activation antigen, B7/BB-1. *Proc. Natl. Acad. Sci. USA.* 87:5031.
9. Hansen, J.A., P.J. Martin, and R.C. Nowinski. 1980. Monoclonal antibodies identifying a novel T-cell antigen and Ia antigens of human lymphocytes. *Immunogenetics.* 10:247.
10. Hara, T., S.M. Fu, and J.A. Hansen. 1985. Human T cell activation. II. A new activation pathway used by a major T cell population via a disulfide-bonded dimer of a 44-kilodalton polypeptide (9.3 antigen). *J. Exp. Med.* 161:1513.
11. Linsley, P.S., W. Brady, L. Grosmaire, A. Aruffo, N.K. Damle, and J.A. Ledbetter. 1991. Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. *J. Exp. Med.* 173:721.
12. Gimmi, C.D., G.J. Freeman, J.G. Gribben, K. Sugita, A.S. Freedman, C. Morimoto, and L.M. Nadler. 1991. B-cell surface B7 provides a costimulatory signal that induces T cells to proliferate and secrete interleukin 2. *Proc. Natl. Acad. Sci. USA.* 88:6575.
13. Azuma, M., M. Cayabyab, D. Buck, J.H. Phillips, and L.L. Lanier. 1992. CD28 interaction with B7 costimulates primary allogeneic proliferative responses and cytotoxicity mediated by small, resting T lymphocytes. *J. Exp. Med.* 175:353.
14. Fraser, J.D., B.A. Irving, G.R. Crabtree, and A. Weiss. 1991. Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. *Science (Wash. DC).* 251:313.
15. Lindsten, T., C.H. June, J.A. Ledbetter, G. Stella, and C.B. Thompson. 1989. Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway. *Science (Wash. DC).* 244:339.
16. June, C.H., J.A. Ledbetter, M.M. Gillespie, T. Linsten, and C.B. Thompson. 1987. T-cell proliferation involving the CD28 pathway is associated with cyclosporine-resistant interleukin 2 gene expression. *Mol. Cell. Biol.* 7:4472.
17. Valle, A., P. Garrone, H. Yssel, J.Y. Bonnefoy, A.S. Freedman, G. Freeman, L.M. Nadler, and J. Banchemereau. 1990. mAb 104, a new monoclonal antibody, recognizes the B7 antigen that is expressed on activated B cells and HTLV-1-transformed T cells. *Immunology.* 69:531.
18. Yssel, H., J.E. De Vries, M. Koken, W. van Blitterswijk, and H. Spits. 1984. Serum-free medium for the generation and the propagation of functional human cytotoxic and helper T cell clones. *J. Immunol. Methods.* 72:219.
19. Yssel, H., D. Blanchard, A. Boylston, J. de Vries, and H. Spits. 1987. T cell clones which share T cell receptor epitopes differ in phenotype, function, and specificity. *Eur. J. Immunol.* 16:1187.
20. Haanen, J.B., R. de Waal Malefyt, P.C. Res, E.M. Kraakman, T.H. Ottenhoff, R.R. de Vries, and H. Spits. 1991. Selection of a human T helper 1-like subset by mycobacteria. *J. Exp. Med.* 174:583.
21. Yssel, H., K.E. Johnson, P.V. Schneider, J. Wideman, A. Terr, R. Kastelein, and J.E. de Vries. 1992. T cell activation-inducing epitopes of the house dust mite allergen *Der p 1*. Proliferation and lymphokine production patterns by *Der p 1*-specific CD4⁺ T cell clones. *J. Immunol.* 148:738.
22. de Waal Malefyt, R., S. Verma, M.-T. Bejarano, M. Ranes-Goldberg, M. Hill, and H. Spits. 1993. CD2/LFA-3 or LFA-1/ICAM-1 but not CD28/B7 interactions can augment cytotoxicity by virus specific CD8⁺ CTL. *Eur. J. Immunol.* In press.
23. Lanier, L.L., and D.J. Recktenwald. 1991. Multicolor immunofluorescence and flow cytometry. *Methods: A Companion to Methods Enzymol.* 2:192.
24. Azuma, M., M. Cayabyab, D. Buck, J.H. Phillips, and L.L. Lanier. 1992. Involvement of CD28 in major histocompatibility complex-unrestricted cytotoxicity mediated by a human NK leukemia cell line. *J. Immunol.* 149:1115.
25. Lanier, L.L., N.A. Federspiel, J.J. Ruitenber, J.H. Phillips, J.P. Allison, D. Littman, and A. Weiss. 1987. The T cell antigen receptor complex expressed on normal peripheral blood CD4⁻CD8⁻ T lymphocytes: a CD3-associated disulfide-linked γ chain heterodimer. *J. Exp. Med.* 165:1076.
26. Lanier, L.L., G. Yu, and J.H. Phillips. 1989. Co-association of CD3 ζ with a receptor (CD16) for IgG Fc on human natural killer cells. *Nature (Lond.).* 342:803.

27. Norton, S.D., L. Zuckerman, K.B. Urdahl, R. Shefner, J. Miller, and M.K. Jenkins. 1992. The CD28 ligand, B7, enhances IL-2 production by providing costimulatory signal to T cells. *J. Immunol.* 149:1556.
28. Gerrard, T.L., D.J. Volkman, C.H. Jurgensen, and A.S. Fauci. 1986. Activated human T cells can present denatured antigen. *Hum. Immunol.* 17:416.
29. Roncarolo, M.-G., M. Bigler, J.B.A. Haanen, H. Yssel, R. Bacchetta, J.E. de Vries, and H. Spits. 1991. Natural killer cell clones can efficiently process and present protein antigens. *J. Immunol.* 147:781.
30. Favre, C., J. Wijdenes, H. Cabrillat, O. Djossou, J. Banchereau, and J.E. de Vries. 1989. Epitope mapping of recombinant human gamma interferon using monoclonal antibodies. *Mol. Immunol.* 26:17.
31. Sansom, D.M., and N.D. Hall. 1993. B7/BBI, the ligand for CD28, is expressed on repeatedly activated human T cells *in vitro*. *Eur. J. Immunol.* 23:295.