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The BB1 Monoclonal Antibody Recognizes Both Cell Surface CD74 (MHC Class II-Associated Invariant Chain) as Well as B7-1 (CD80), Resolving the Question Regarding a Third CD28/CTLA-4 Counterreceptor[1]

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The identification of all CD28/CTLA-4 counterreceptors is critical to our understanding of this pivotal pathway of T cell activation. Clouding our understanding has been the reported discrepancies in expression and function of the B7-1 (CD80) molecule based upon the use of the BB1 vs other anti-B7-1 mAbs. To resolve this issue, we have cloned a BB1-binding molecule from the BB1$^+$B7-1$^-$ NALM-6 pre-B cell line. Here, we demonstrate that this BB1-binding molecule is identical to the cell surface form of CD74 (MHC class II-associated invariant chain). CD74-transfected cells bound the BB1 mAb but not other anti-CD80 mAbs, CD28-Ig, or CTLA4Ig. Absorption and blocking experiments confirmed the reactivity of BB1 mAb with CD74. A region of weak homology was identified between CD74 and the region of B7-1 encoding the BB1 epitope. Therefore, the BB1 mAb binds to a protein distinct from B7-1, and this epitope is also present on the B7-1 protein. Many of the puzzling observations in the literature concerning the expression of human B7-1 are resolved by an understanding that BB1 staining is the summation of CD74 plus B7-1 expression. This observation requires the field to reconsider studies using BB1 mAb in the analysis of CD80 expression and function. *The Journal of Immunology,* **1998, 161: 2708–2715.**

Ever mounting evidence supports the notion that signaling via the T cell surface molecules CD28 and CTLA-4 is central to the regulation of T cell activation (reviewed in Ref. 1). Moreover, these pathways have important clinical implications in the pathogenesis and treatment of human disease. Therefore, the identification as well as the molecular, phenotypic, and functional characterization of all CD28 and CTLA-4 counterreceptors will be critical to our understanding and manipulation of this pathway.

Shortly after the discovery that the B7-1 molecule was a high affinity ligand for CTLA-4 and low affinity ligand for CD28 (2), several lines of evidence suggested that additional CD28/CTLA-4 counterreceptors existed. The B7-1 (CD80) molecule was initially defined by two mAb termed 133 (B7-1) and BB1 (3, 4). These mAb both bound to B7-1 transfectants, cross-blocked each other's binding, and blocked CD28-mediated costimulation, indicating that they both bound to B7-1. Of note, the BB1 mAb was instrumental in the discovery that CD28 is a counterreceptor for B7-1 (2), and indeed for several years B7-1 was often referred to in the literature as B7/BB1. In spite of the common features shared by the B7-1 and BB1 mAbs, a number of conflicting observations were reported. As noted in our initial characterization of B7, the B7-1 and BB1 mAbs consistently differed in their binding to some cell lines (3). From our studies of CTLA4Ig binding to activated B cells, we proposed the existence of a second CTLA-4 counterreceptor that was present on B cells early following activation and did not stain with either anti-B7-1 or BB1 mAbs (5). These observations culminated in the cloning of B7-2, a second CD28/CTLA-4 counterreceptor (6, 7). B7-2 transfectants did not stain with either B7-1 or BB1 mAbs but did bind both CD28-Ig and CTLA4Ig (6). We also identified a population of activated B cells that did not stain with anti-B7-1 but did stain with the BB1 mAb and CTLA4Ig (5). We termed this molecule B7-3.

Although the discovery of B7-2 as well as the clustering of B7-1 into the CD80 cluster (8) and B7-2 into the CD86 cluster (9) significantly advanced our understanding of CD28 and CTLA-4 counterreceptors, the earlier phenotypic and functional studies with anti-B7-1 and BB1 Abs still suggested that another molecule might exist. In fact, the Fifth International Workshop in 1993 reported that, by cellular expression and functional analysis, it was not possible to exclude the existence of a distinct molecule (8). While clearly reacting with B7-1 protein, the BB1 mAb has been shown to differ from other anti-B7-1 mAbs in Western blotting (10), chromosomal location (11, 12), and reactivity with thymic epithelial cells (13). Activated keratinocytes bound the BB1 Ab but not other B7-1 mAbs and did not express B7-1 mRNA (13). Surprisingly, activated keratinocytes were reported to adhere to CD28 transfectants. These studies further supported our previous concern that the BB1 mAb was binding to an additional molecule. Therefore, our working hypothesis was that the BB1 mAb bound to an epitope on a distinct protein and that this epitope was also expressed on the B7-1 protein.

In the present report, we have cloned a BB1-binding molecule distinct from CD80 and demonstrate its identity to the cell surface form of CD74, MHC class II-associated invariant chain. Many of

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the puzzling observations in the literature concerning the expression of B7-1 are resolved by an understanding that BB1 staining is the summation of CD74 plus B7-1 expression. Since CD74 is coordinately expressed with MHC class II, staining with BB1 mAb has given the impression that B7-1 is more coordinately expressed with MHC class II than is actually the case. This observation requires that the field reconsider the clustering of anti-B7-1 and BB1 mAbs into the CD80 cluster and refine its characterization of the expression of B7-1.

Materials and Methods

Monoclonal Abs and fusion proteins

mAbs were used as purified Ig unless otherwise indicated. BB1 mAb (IgM) (4) was prepared by one of us (E.A.C.) and was also purchased as purified and FITC-conjugated BB1 from Ancell (Bayport, MN). The anti-B7-1 mAbs used were: 133 (IgM) (3); EW3.4B2.C4 (IgG2a), EW3.1A5.F3 (IgG2a), and EW3.6B9 (IgG2a) (Repligen, Cambridge, MA); B7g (IgG1), 49 (IgG1), and L307.4 (IgG1, this mAb was sold for a time by Becton Dickinson as "BB1" but has no relation to the original BB1 mAb and was generated by immunization with B7-1 transfectants) (Leukocyte Typing V Workshop, Boston, MA); and 104 (IgG1; Dr. J. Banchereau, Dardilly, France) (14). The anti-B7-2 mAb used was HA3.1F9 (IgG1, Repligen) (15). Anti-CD74 mAbs LN2 (IgG1), M-B741 (IgG2a), and M-B741-FITC were obtained from PharMingen (San Diego, CA). CD28-Ig was a generous gift from Dr. P. Linsley (Bristol-Myers Squibb, Seattle, WA) (16). CTLA4Ig and control Ig fusion proteins were obtained from Repligen. Other mAbs used include anti-MHC I (W6/32; IgG2a), anti-CD11a (Mo1; IgM), anti-CD14 (Mo2; IgM), and anti-CD56 (3B8; IgM). For flow cytometric analysis, where indicated, fluorochrome-conjugated mAbs were prepared. FITC-conjugated anti-CD10 (J5; IgG2a) was obtained from Coulter (Hialeah, FL). FITC-conjugated goat anti-mouse IgG and IgM, FITC-conjugated goat anti-human IgG, and FITC-conjugated streptavidin were purchased from Southern Biotechnology (Birmingham, AL).

Phenotypic analysis

Expression of cell surface molecules was determined by direct or indirect labeling using standard methodology. For lymphocytes, Fc receptors were blocked by incubation with mouse Ig (for direct staining) or goat Ig (for indirect staining) before the addition of the specific mAbs. Irrelevant isotype-matched Abs (IgG subclasses or IgM) or control fusion protein were used as negative controls. FITC-conjugated goat anti-mouse IgG or IgM and FITC-conjugated goat anti-human IgG were used for unconjugated mAbs and Ig-fusion proteins, respectively. Samples were analyzed in a Coulter Elite flow cytometer.

Ab-binding competition

Cells were incubated with 10 μg/ml of unconjugated competitor mAbs for 30 min at 4°C, washed twice with PBS/0.1% BSA, and then incubated with FITC-conjugated CD74, CD10, or CD19. After 30 min incubation at 4°C, the cells were washed once and analyzed by flow cytometry.

Ab absorption

BB1 mAb (0.5 ml of 10 μg/ml) was absorbed by incubation with 5×10^6 of either B7-1-transfected Chinese hamster ovary (CHO)[3] cells (CHO/B7-1) or Neo vector-transfected CHO cells (CHO/Neo) (17) for 30 min at 4°C. Cells were pelleted by centrifugation and the absorption repeated with a fresh 5×10^6 cells.

cDNA library

RNA was prepared from NALM-6 cells and oligo(dT) selected twice. An oriented cDNA library was constructed in the pAXEF vector with an oligo(dT_{15})-Not primer and *Sal*I adapter using the superscript cDNA synthesis kit according to the manufacturer's instructions (Life Technologies, Gaithersburg, MD). The pAXEF mammalian expression vector contains the elongation factor 1α (EF1) promoter and intron, multiple cloning site, supF, β-globin polyadenylation site, SV40 origin, and ColEI origin. It was constructed using DNA fragments from pEF-BOS (18), p142B, and pAX114 (19), and the sequence is available upon request. The library was introduced into DH10B/P3 *Escherichia coli*, and a total of 7×10^8 independent transformants were generated.

[3] Abbreviations used in this paper: CHO, Chinese hamster ovary; ALL, acute lymphoblastic leukemia.

COS cell transfection and molecular cloning

For the first round of selection, COS cells were transfected via the DEAE-Dextran procedure (20) with 0.2 μg of NALM-6 plasmid library DNA per 100-mm dish. After 40 h, cells were harvested, incubated with 5 μg/ml BB1 mAb, washed, and panned on anti-IgM-coated plates as described (20, 21). Episomal DNA was prepared from adherent cells, reintroduced into *E. coli*, and transfected into COS cells by polyethylene glycol-mediated fusion of spheroplasts (20). The panning with BB1 mAb was repeated. Individual plasmid DNAs were transfected into COS cells via the DEAE-Dextran procedure (4 μg per 100-mm dish) and analyzed after 72 h for cell surface expression by indirect immunofluorescence and flow cytometry.

RNA blot hybridizations

RNA was prepared and analyzed by blot hybridization as previously described (22). The 1.3-kb CD74 cDNA insert was labeled by random oligonucleotide priming using α-^{32}P-labeled dCTP and the Klenow fragment of DNA polymerase. Hybridization, washing, and autoradiography were performed as previously described (22).

Keratinocytes

Normal human keratinocytes were derived from neonatal foreskins by trypsinization, were cultured in low calcium (0.15 mM) keratinocyte growth medium (Clonetics, San Diego, CA), and were used at passages 2 to 4 at 70 to 80% confluency. Keratinocytes were treated with 100 U/ml IFN-γ (Genzyme, Cambridge, MA) or 10 ng/ml PMA (Sigma, St. Louis, MO).

B cell precursor acute lymphoblastic leukemia samples

B cell precursor ALL cells (pre-B ALL) were obtained from the bone marrow of patients with leukemia with greater than 90% marrow infiltration. Both fresh and cryopreserved samples were used in these studies. Appropriate informed consent and Institutional Review Board approval were obtained for all sample collections. Samples were enriched by density centrifugation over Ficoll-Hypaque, then washed twice in RPMI 1640 supplemented with 10% FCS and 2 mM L-glutamine.

Stimulation of B cell precursor ALL cells by CD40 cross-linking

National Institutes of Health-3T3 cells stably transfected with human CD40 ligand (t-CD40L) or vector (t-mock), have been described (23, 24). The t-CD40L or t-mock cells were irradiated at 96 Gy and plated at 1×10^5 cells/well in 24-well plates or 4×10^5 cells/well in 6-well plates, in media without G418, and incubated overnight at 37°C in a 5% CO_2 humidified atmosphere. The plates were washed twice with PBS, and pre-B ALL cells were added at 5×10^5 cells/ml in IMDM supplemented with 2% FCS, 0.5 mg/ml BSA, 2 mM L-glutamine, 50 μg/ml iron-saturated transferrin, 5 μg/ml bovine insulin, and 2 ng/ml of recombinant human IL-4 (a gift of Immunex, Seattle, WA). On day 5, CD40-stimulated pre-B ALL cells were harvested, washed in IMDM, and used for phenotypic analysis.

Results

Most pre-B ALL cells bind the BB1 mAb but do not express B7-1, phenotypic evidence that the BB1 mAb has dual specificity for B7-1 and an additional protein

The CD80 cluster was defined in the Fifth International Workshop on Leukocyte Antigens on the basis of the reactivity of CHO/B7-1 with five mAbs (8) including the original B7-1 mAb (clone 133) (3) and the BB1 mAb (4). Nevertheless, in the original characterization of the B7-1 mAb, we had distinguished B7-1 mAb from BB1 by the capacity of BB1 but not B7-1 to stain the B cell line, BALM4 (3). One possible source of this different reactivity is that the immunogen used to generate the BB1 mAb was a baboon B cell line (4), and, consequently, the binding site might not precisely fit the human B7-1 molecule in the same way as mAb generated using human B7-1. Furthermore, BB1 mAb is an IgM and presumably has only a low affinity since it has not undergone affinity maturation.

To resolve the controversy of whether the BB1 mAb recognizes an additional protein, we analyzed an additional pre-B cell line and primary pre-B cell tumors. We observed that the pre-B cell line NALM-6 and 10 of 12 pre-B ALL stained positively with the BB1

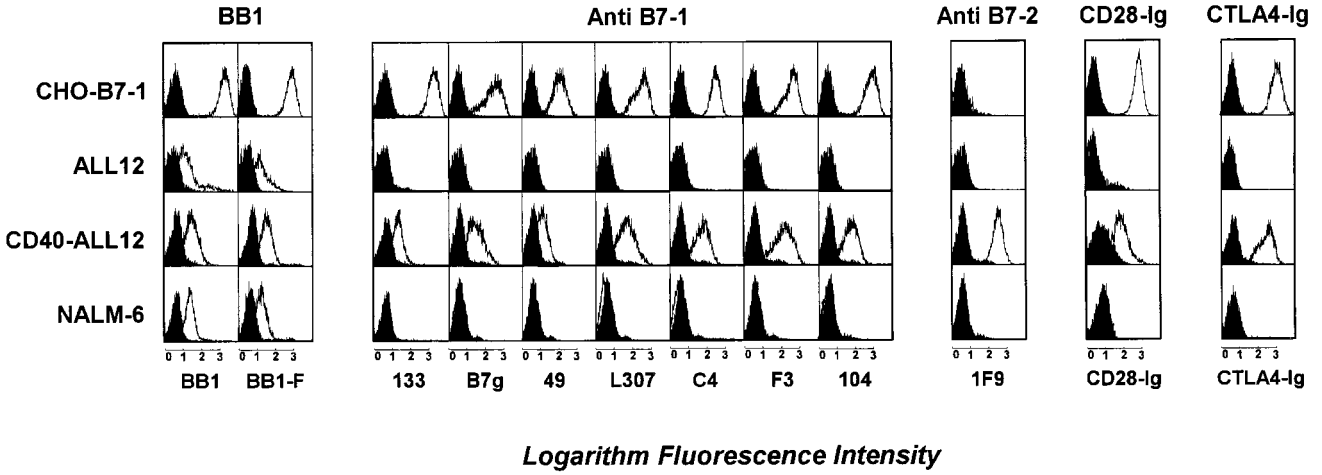

FIGURE 1. BB1 mAb but not B7-1 mAbs react with pre-B cell ALL. The cells indicated on the *left* were stained with the mAbs or Ig fusion proteins indicated at the *bottom* and analyzed by flow cytometry. CHO/B7-1 cells have been stably transfected with B7-1 cDNA. ALL12 indicates unstimulated cells from ALL patient 12, and CD40-ALL12 indicates CD40-stimulated cells from patient 12. NALM-6 is a pre-B cell line. BB1 Ab was used in both the purified (BB1) and FITC-conjugated (BB1-F) forms. Unfilled histograms represent the fluorescence distribution with the indicated mAbs, and filled histograms represent that of isotype-matched control Abs. The *y*-axis indicates cell number.

mAb, although none of them stained with the four other mAbs from the CD80 panel of the Fifth Workshop (133, B7g, 49, L307) or other anti-B7-1 mAbs (EW3.4B2.C4, EW3.1A5.F3, 104). The BB1 mAb consistently stained pre-B ALL and NALM-6 cells with low to intermediate intensity (range 29–85% positive cells for pre-B ALL samples). Cell staining profiles for a representative pre-B ALL (ALL-12) are shown in Figure 1. CHO/B7-1 cells were also stained as positive controls and reacted with the BB1 mAb and all seven anti-B7-1 mAbs, confirming that BB1 mAb does indeed recognize the B7-1 molecule. CHO/B7-1 cells bound CD28-Ig and CTLA4Ig but did not stain with anti-B7-2 mAb. Although approximately 60% of pre-B ALL samples stain with anti-B7-2 mAb, both ALL-12 and NALM-6 did not. ALL-12 and NALM-6 bound BB1 but did not bind any of the other seven B7-1 mAb. In contrast to CHO/B7-1, neither ALL-12 nor NALM-6 bound CD28-Ig or CTLA4Ig. CD40 activation of ALL 12, known to induce B7-1 and B7-2 expression, resulted in binding to BB1, all seven B7-1 mAbs, anti-B7-2, and both CD28-Ig and CTLA4Ig. These findings demonstrate that the reactivity of BB1 mAb was distinct from the staining profile observed with other anti-B7-1 mAbs. Moreover, they demonstrate that the additional BB1-binding molecule expressed on ALL 12 and NALM-6 is not a CD28 or CTLA-4 counterreceptor.

Molecular confirmation of the above results is derived from our previous observation that pre-B ALL do not express B7-1 mRNA (21), and this was confirmed for these samples by RT-PCR (data not shown). In addition, no cytoplasmic B7-1 Ag was detected in pre-B ALL or NALM-6 cells by flow cytometry nor was any B7-1 detected by immunoprecipitation with anti-B7-1 mAb (clone EW3.4B2.C4), though B7-1 was readily immunoprecipitated from CD40-stimulated pre-B ALL and CHO/B7-1 (data not shown).

Molecular cloning with BB1 mAb demonstrates that the BB1 mAb also recognizes CD74

A cDNA expression library was prepared from $BB1^{+}B7\text{-}1^{-}$ NALM-6 cells and transfected into COS cells. The BB1 mAb was used to immunoselect a BB1 cDNA. Multiple copies of a 1.3-kb cDNA were isolated. Sequencing and data base search of this 1.3-kb cDNA revealed it to encode CD74, the invariant chain of the MHC class II Ag presentation pathway (GenBank accession number X00497; Ref. 25). While primarily an intracellular protein, some CD74 is transported to the cell surface (26–28). COS cells transfected with this CD74 cDNA stained with the BB1 mAb, but not the original anti-B7-1 mAb (clone 133) (Fig. 2) or other anti-B7-1 mAbs (data not shown). The reactivity of BB1 mAb with CD74 was found with all samples of BB1 tested, including five different samples of BB1 produced in the laboratory of E.A.C., the BB1 from the Fifth Workshop, and commercially produced BB1 (Ancell) (data not shown). COS cells transfected with B7-1 cDNA bound both BB1 and the 133 mAbs, confirming the dual specificity

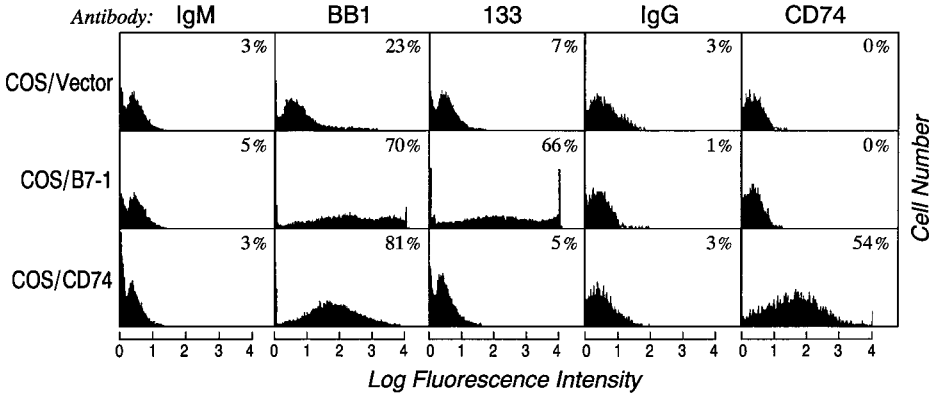

FIGURE 2. Reactivity of BB1 mAb with both B7-1- and CD74-transfected COS cells. COS cells were transfected with the cDNA clones indicated on the *left*, stained with the mAbs indicated *above* each panel, and analyzed by flow cytometry. The percentage of positively stained cells is indicated in the *upper right*.

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mB7-1    81 - IAGKLKVWPEYKNRTLYDNT TYSLIILGLVLSDRGTYSCVVQ - 122
              * | * *|||||||||**| |   |**|| |  || ||| |||
hB7-1    77 - MSGDMNIWPEYKNRTIFDITNNLSIVILALRPSDEGTYECVVL - 119
                 *   |  *  *    *   *|   |  *| |    | * |
 CD74   171 - NTMETIDWKVFESWMHHWLLFEMSRHSLEQKPTDAPPKESLEL - 213
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FIGURE 3. Sequence comparison of mB7-1, hB7-1, and hCD74. The IgV domain of B7-1 was aligned with the extracellular domain of CD74 using the Lawrence comparison routine and the Dayhoff scoring matrix of Eugene (58). Amino acids are numbered with the initial methionine as 1. Vertical lines (|) indicate identity and stars (*) indicate similar amino acids. The amino acids in human B7-1, whose mutation eliminates BB1 binding, are underlined (30).

of the BB1 mAb (Fig. 2). Cell surface expression of CD74 was demonstrated on CD74-transfected COS cells using a CD74 mAb. The CD74 mAb did not stain B7-1-transfected COS cells.

We were surprised to find that CD74 contains a BB1 epitope since CD74 and B7-1 are dissimilar structures. B7-1 is a type I membrane protein with an extracellular region consisting of Ig-V- and Ig-C-like domains. The BB1 epitope is contained within the Ig-V domain of B7-1 (29). Mutation of a pair of amino acids in the CDR2 region of the Ig-V domain from MSGD (77–80) to ASGN abolishes BB1 binding (30). Murine and human B7-1 are poorly conserved in the CDR2 loop containing the BB1 epitope (Fig. 3), with a charge shift from aspartic acid to lysine at amino acid 80 probably accounting for the antigenicity of the human epitope in mice. In contrast, CD74 is a type II membrane protein. The carboxyl-terminal 160 amino acids (amino acids 73–232) comprise the extracellular domain and do not contain the characteristic sequence motifs of an Ig family member but instead contain a high proportion of α helical structure. Nevertheless, sequence comparison of B7-1 and CD74 identifies a region of weak homology that includes the region comprising the BB1 epitope. This alignment of CD74 conservatively substitutes a glutamic acid for aspartic acid at position 80 of human B7-1, is identical to B7-1 at tryptophan 84, and conservatively substitutes phenylalanine for tyrosine at position 87. Provocatively, tryptophan 84 and tyrosine 87 have been shown to be the most critical amino acids for binding of the B7-1 Ig-V domain to CD28 (30, 31). Whether this is the region of CD74 that folds into a three-dimensional structure comprising a BB1 epitope is uncertain.

CD74-transfected COS cells do not bind CD28-Ig or CTLA4Ig

Since $BB1^{+}B7\text{-}1^{-}$ keratinocytes have been reported to bind to CD28-transfected CHO cells, we examined the binding of CD28-Ig and CTLA4Ig to CD74-transfected COS cells. Neither CD28-Ig or CTLA4Ig bound to CD74-transfected COS cells though both bound to B7-1-transfected COS cells (Fig. 4*A*). The failure of CTLA4Ig to bind to CD74-transfected COS cells also eliminates the possibility that CD74 transfection induces expression of the endogenous B7-1 of African green monkey COS cells, which would bind both BB1 and CTLA4Ig. In addition, CD74-transfected COS cells did not bind other B7-1 or B7-2 mAbs, which have been shown to bind monkey B7-1 or B7-2 (32) (data not shown).

The CD74 reactivity of BB1 is absorbed by CHO/B7-1

To examine whether the BB1 mAb preparation might contain separate Abs to B7-1 and CD74, we examined whether absorption of BB1 on CHO/B7-1 cells removed its CD74 reactivity. BB1 absorbed on CHO/B7-1 cells did not stain either CD74 or B7-1-transfected COS cells, demonstrating that the same BB1 mAb recognizes both structures (Fig. 4*B*). Absorption of BB1 on CHO/Neo cells did not affect reactivity of BB1 with either B7-1 or CD74.

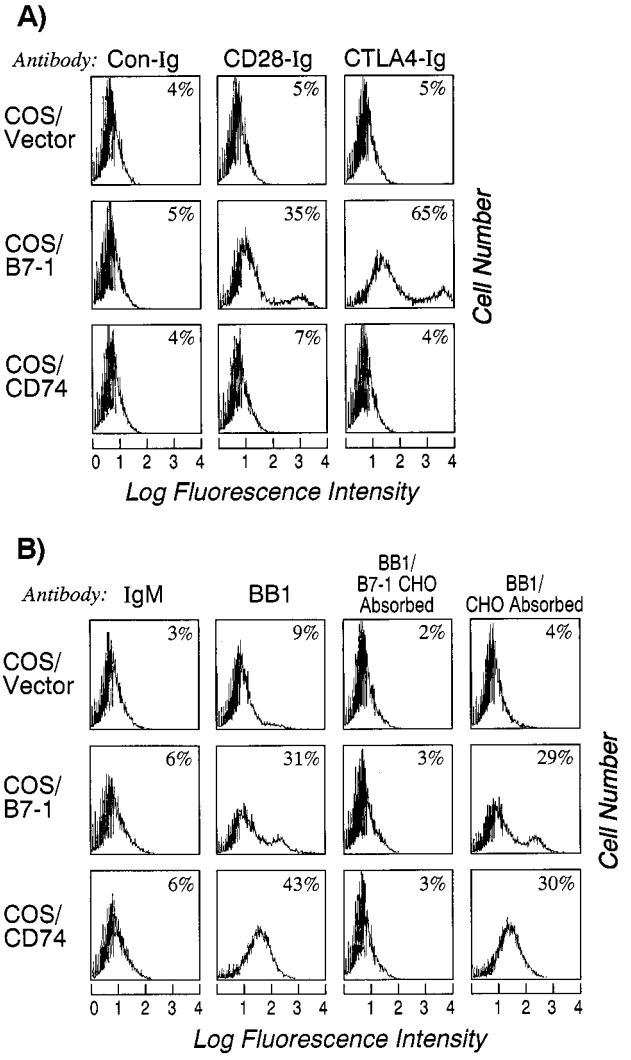

FIGURE 4. Reactivity of CD74-transfected COS cells with (*A*) CD28-Ig and CTLA4Ig and with (*B*) BB1 mAb after absorption on CHO/B7-1. COS cells were transfected with the cDNA clones indicated on the *left*, stained with mAbs or Ig fusion proteins indicated *above* each panel, and analyzed by flow cytometry. BB1 mAb was absorbed with CHO/B7-1 cells or CHO/Neo cells as described in *Materials and Methods*. The percentage of positively stained cells is indicated in the *upper right*.

Anti-CD74 mAb binding to CD74 is blocked by BB1 mAb

To determine whether the BB1 binding seen on NALM-6 cells is to CD74, we examined the capacity of the BB1 mAb to block anti-CD74 mAb binding. Figure 5*A* shows that the BB1 and anti-CD74 mAbs, as well as control anti-CD10 and anti-CD19 mAbs, all bind to NALM-6 cells. Preincubation of NALM-6 cells with BB1 mAb blocks binding of anti-CD74 mAb but not anti-CD10 or anti-CD19 mAbs (Fig. 5B). In contrast, preincubation with control IgM did not affect the binding of anti-CD74, anti-CD10, or anti-CD19 mAbs (Fig. 5*C*). The inhibitory effect of BB1 on anti-CD74 binding was specific since preincubation with other IgM mAbs, 3B8 (anti-CD56), Mo1 (anti-CD11b), or Mo2 (anti-CD14) did not block binding of anti-CD74 mAb (Fig. 5*D*).

IFN-γ induces CD74 mRNA expression in keratinocytes

Keratinocytes stimulated with IFN-γ have been shown to bind BB1 mAb but not to bind other anti-B7-1 mAbs or express B7-1 mRNA (13, 33). Figure 6 shows that untreated or PMA-treated

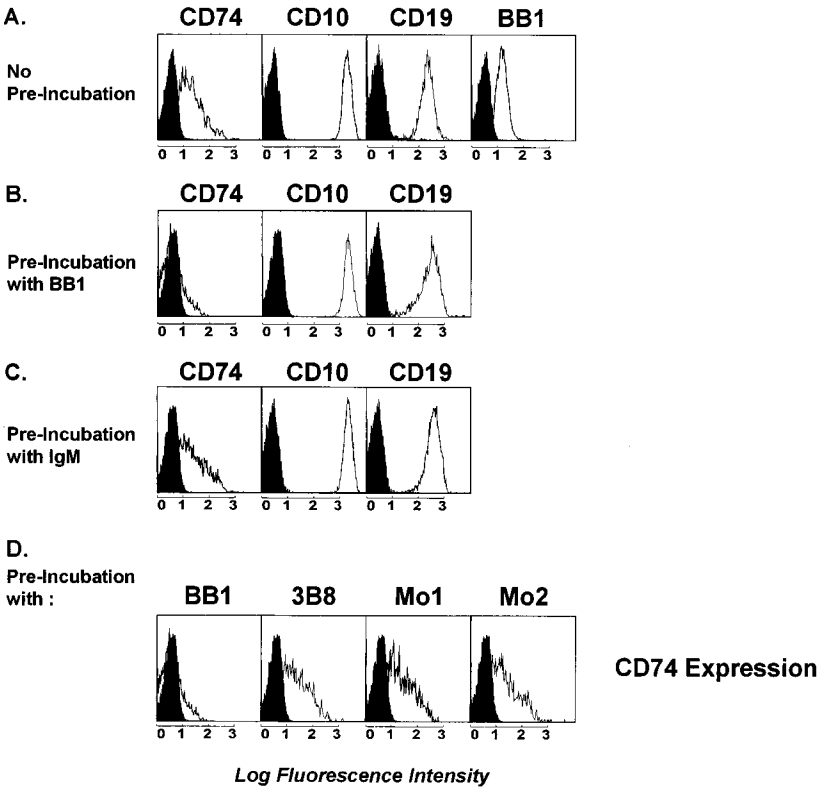

FIGURE 5. BB1 blocks the binding of CD74 mAb to NALM-6 cells. *A*, Control staining of NALM-6 cells with FITC-conjugated CD74, CD10, CD19, and BB1 mAbs. *B*, Staining of NALM-6 cells with FITC-conjugated CD74, CD10, and CD19 mAbs following preincubation with 10 μg/ml BB1 mAb. *C*, Staining of NALM-6 cells with FITC-conjugated CD74, CD10, and CD19 mAbs following preincubation with 10 μg/ml control irrelevant murine IgM. *D*, Staining of NALM-6 cells with FITC-conjugated CD74 mAb following preincubation with 10 μg/ml of the isotype-matched (IgM) Abs BB1, 3B8 (anti-CD56), Mo1 (anti-CD11b), or Mo2 (anti-CD14). Unfilled histograms represent the fluorescence distribution with the indicated mAbs, and filled histograms represent that of isotype-matched FITC-conjugated control Abs. The *y*-axis indicates cell number.

keratinocytes do not express CD74 mRNA but that IFN-γ induces keratinocytes to express CD74 mRNA weakly by 8 h and strongly by 24 h. CD74 mRNA is also strongly expressed in NALM-6, Raji, and BALM4 cells. Indeed, the binding of the BB1 but not the B7-1 mAb to the BALM4 cell line was the original basis for distinguishing BB1 from B7-1 (3).

Discussion

In the present report, we demonstrate that a BB1-binding molecule that is expressed on pre-B ALL cells is distinct from B7-1. This molecule is not a CD28 or CTLA-4 counterreceptor. Molecular cloning demonstrated that CD74 was a second protein that reacted with BB1 mAb. Therefore, our hypothesis (5) that the BB1 mAb bound to an epitope on a distinct BB1 protein and that this epitope was also expressed on the B7-1 protein is correct.

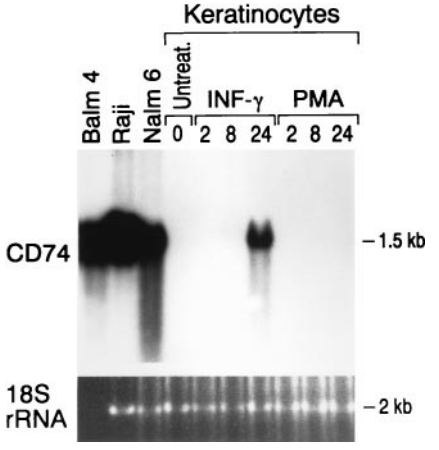

FIGURE 6. IFN-γ induces CD74 mRNA expression in keratinocytes. RNA was prepared from the pre-B cell lines BALM4 and NALM-6 and the Burkit's lymphoma line, Raji. RNA was prepared from unstimulated keratinocytes or at 2, 8, and 24 h after stimulation with IFN-γ or PMA. Twenty micrograms of the indicated RNA was loaded on each lane except for BALM4, in which 1 μg of poly(A)$^+$ RNA was loaded. The gel was blotted and hybridized with the CD74 cDNA. The *lower panel* shows the gel after staining with ethidium bromide to demonstrate equivalent loading of gel lanes.

There have been multiple reported molecular and phenotypic discrepancies depending on the use of BB1 mAb or other anti-B7-1 mAbs. Examination of these discrepancies shows that they are resolved by an understanding that BB1 staining is the summation of CD74 plus B7-1 expression. BB1 was first reported to Western blot a 37-kDa protein (34), more consistent with the 33 to 43 kDa range of CD74 isoforms than with the 44- to 70-kDa range of B7-1 (14, 21). The first reported phenotypic discrepancy, that BB1 mAb but not anti-B7-1 mAb (clone 133) binds to BALM-4 cells (3), is clearly resolved by the expression of CD74 on BALM-4 cells.

Turka et al. reported that BB1 stained thymic interlobular fibrous septa and some thymic epithelial cells (13, 35) whereas two reports using other anti-B7-1 mAbs both found B7-1 expression on scattered, mostly medullary cells with a dendritic or macrophage morphology but not on thymic epithelial cells or thymic interlobular fibrous septa (36, 37). The expression of the BB1 epitope on MHC class II$^+$ thymic epithelial cells presumably reflects expression of CD74. The expression of MHC class II, CD74, and HLA-DM genes are coordinately controlled by the class II transcriptional *trans*-activator protein (CIITA) via shared sequence motifs in their promoter regions (38, 39). CIITA regulates both constitutive and inducible expression (40). Since CD74 is coexpressed with MHC class II, staining with BB1 mAb has given the impression that B7-1 is more coordinately expressed with MHC class II than is actually the case.

We reported that anti-Ig-activated human B cells expressed a CTLA-4 counterreceptor by 24 h, whereas the binding of BB1 and B7-1 mAbs was observed only after 48 h (5). Further work identified

this early CTLA-4 counterreceptor as B7-2 (CD86) (6). B7-2-transfected cells were not stained by BB1, and their costimulatory activity was blocked by CTLA4Ig but not BB1 mAb (6). When 72-h-activated B cells were sorted into B7-1$^+$ and B7-1$^-$ populations, the B7-1$^-$-activated B cells bound both BB1 mAb and CTLA4Ig. These B7-1$^-$-activated B cells modestly costimulated T cell proliferation, which was blocked by BB1 mAb, CD28 Fab, and CTLA4Ig but not by B7-1 mAb. Therefore, we hypothesized the existence of a third CD28/CTLA-4 counterreceptor, termed B7-3, that was capable of inducing modest T cell proliferation. Based on the the development of mAbs to B7-2 as well as the results of the present study, we now believe that the phenotypic and costimulatory characteristics of the B7-1$^-$-activated B cells were due to the combined expression on B7-1$^-$-activated B cells of B7-2, a CD28/CTLA-4 counterreceptor, and CD74, a BB1-binding molecule. Therefore, the hypothesized B7-3 is a BB1-binding molecule but is not a third CD28/CTLA-4 counterreceptor but rather a cross-reactive epitope on CD74.

Kulova et al., using the BB1 mAb, reported that, after MHC class II ligation of dense tonsillar B cells, B7-1 expression increased rapidly, peaked within 6 to 9 h, and then waned (41). In contrast, Boussiotis et al., using the B7-1 mAb, found that cross-linking MHC class II on resting splenic B cells induced B7-1 expression only after 48 h (5). These differences may be due to differences in the cell sources used (tonsillar B cells tend to be more activated than splenic B cells) or could be due to Koulova et al. detecting a rapid increase in CD74 expression using the BB1 mAb. It will be of interest to determine which B cell activation signals regulate CD74 expression and whether increases in cell surface CD74 reflect changes in Ag processing or presentation pathways. What is clear is that ligation of MHC class II does increase B7-1 expression (5, 42), implying that MHC class II and B7-1, as might be expected, work closely together to activate T cells via TCR and CD28.

Multiple investigators have reported that BB1 is not expressed on unstimulated keratinocytes but is induced by IFN-γ (reviewed in Ref. 43). However, no B7-1 epitopes are expressed nor is B7-1 mRNA detectable (13, 33). IFN-γ induces MHC class II on keratinocytes (44), and MHC class II and CD74 have been shown to be expressed by keratinocytes during the tuberculin reaction and in diseases such as lichen planus (45, 46). We show here that IFN-γ induces CD74 mRNA in keratinocytes. Strikingly, immunohistochemical analysis of keratinocytes showed that most of the BB1 staining was intracellular (13), as would be expected for CD74 present in intracellular Ag-processing compartments. Indeed, we have found that BB1 mAb exhibits strong intracellular staining of BB1$^+$B7-1$^-$ pre-B ALL cells (data not shown). This leads to the conclusion that the BB1 epitope expressed on keratinocytes and pre-B ALL cells is on CD74. When BB1 mAb is used for immunohistochemistry, which can detect both intracellular and cell surface Ag, the contribution of CD74 to the total staining would be greater than when BB1 is used for cell surface analysis by flow cytometry. Nickoloff et al. also found that BB1$^+$ keratinocytes adhere to CD28-transfected CHO cells, and this was blocked by BB1 mAb, suggesting that the BB1 Ag on keratinocytes is a CD28 counterreceptor. Nevertheless, BB1 mAb did not block the capacity of keratinocytes to costimulate T cells (13). We do not find that CD74-transfected cells bind CD28-Ig or CTLA4Ig though we cannot exclude a very low affinity interaction. CD74 has been reported to act as a costimulatory molecule via binding of CD44 to chondroitin sulfate linked to CD74 (47). The binding of BB1 mAb to CD74 was unaffected by the presence of 100 μg/ml chondroitin sulfate or hyaluronic acid, excluding the possibility that these sugars are involved in BB1 mAb recognition (data not shown). Some of the weak costimulatory activity of B7-1$^-$-activated B cells and IFN-γ-treated keratinocytes might be due to chondroitin sulfate-modified cell surface CD74 binding to CD44 on T cells (47). Alternatively, the BB1 mAb might bind to yet an additional Ag.

The presence of a common structural determinant on both B7-1 and CD74 might not be purely coincidental. At the site of interaction between APC and T cell, critical molecules become clustered in an interaction cap. These have been shown to include TCR, CD4, LFA-1, CD28, and CTLA-4 on the T cell side and MHC class II-peptide, ICAM-1, and, presumably, B7 on the APC side (48–51). The intracellular cytoskeletal protein talin also concentrates beneath the activation cap on the T cell, leading to orientation of the microtubule organizing center and focused delivery of cytokines to the bound APC (48, 49). Perhaps the common structural determinant recognized by the BB1 mAb on both B7-1 and CD74 represents a functionally important region involved in intermolecular interactions between MHC class II and B7-1 that facilitate B7-1 inclusion in the activation cap.

The difficulty in isolating the CD74 cDNA using BB1 mAb prolonged our inability to resolve these discrepancies. CD74 functions to prevent peptide binding to MHC class II, facilitates MHC class II transport, and enhances its localization to Ag-processing compartments (reviewed in Ref. 52). Full-length CD74 cDNA contains two potential translation initiation sites at nucleotides 8–10 and 56–58 whose alternative utilization leads to the p35 and p33 isoforms of CD74, respectively. CD74 is a type II membrane protein, and the major p33 isoform has the NH_2-terminal 31 amino acids intracytoplasmic, a 25-amino acid transmembrane region, and 160 amino acids extracytoplasmic. The CD74 cDNA isolated by expression cloning using the BB1 mAb contains nucleotides 18–1304 of the CD74 sequence (1304 bp, GenBank accession number X00497; Ref. 25). An additional CD74 cDNA beginning at nucleotide 13 was also isolated. Thus, neither cDNA contains the first translation initiation site, and both encode exclusively the p33 isoform of CD74. This was probably critical for the successful BB1 cloning since the 16 additional amino acids found in the p35 but not the p33 isoform contain a signal for retention in the endoplasmic reticulum. In the absence of MHC class II, as is the case for transfected COS cells, CD74 trimers containing the p35 isoform transit poorly from the endosomal compartment and thus have reduced cell surface expression (53). This may explain the considerable difficulty experienced in isolating the BB1/CD74 cDNA by expression cloning since a full-length cDNA would encode both p33 and p35 isoforms and have reduced cell surface expression. Visually, COS cells transfected with the CD74 cDNA become rounded, with massively enlarged vacuolar compartments, as has been reported by others (54, 55). This toxicity makes the production of stable high level CD74 transfectants difficult.

CD74 is produced in molar excess over MHC class II, and, by a poorly understood pathway, some is expressed on the cell surface independent of class II although some cell surface CD74 is associated with MHC class II. Mutant B cell lines deleted for all MHC class II genes still express the same amount of cell surface CD74 (56). The half-life of CD74 on the cell surface is only 3 to 4 min, after which it is internalized (56). In contrast, peptide-MHC class II complexes on the cell surface are long-lived. It has been proposed that cell surface CD74 binds to MHC class II molecules that have lost their antigenic peptide, leading to internalization and reloading with antigenic peptide (28, 56). Binding of BB1 mAb to cell surface CD74 may have effects independent of blockade of B7-1 costimulation. These might include inhibition of Ag presentation or stimulation via cross-linking of associated MHC class II. Somewhat perplexingly, CD74 has been reported to be expressed at low levels on the surface of unstimulated B cells; however, these cells do not bind BB1 mAb (3, 4, 26). This may reflect the inability

of the low affinity BB1 mAb to detect low level CD74 cell surface expression.

While the above results resolve many of the discrepancies concerning the expression of the B7-1 molecule, its temporal relationship to B7-2 expression, and whether a B7-3 exists, we find no supporting evidence for the existence of additional CD28/CTLA-4 counterreceptors. Mice deficient in B7-1 and B7-2 have a severe immune defect, and activated B cells isolated from them do not bind CTLA4Ig (57). Further characterization of these B7-1- and B7-2-deficient mice should definitively test whether additional CD28/CTLA-4 counterreceptors exist in the mouse.

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