2B4, the Natural Killer and T Cell Immunoglobulin Superfamily Surface Protein, Is a Ligand for CD48

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Summary

2B4 is a cell surface glycoprotein related to CD2 and implicated in the regulation of natural killer and T lymphocyte function. A recombinant protein containing the extracellular region of mouse (m)2B4 attached to avidin-coated fluorescent beads bound to rodent cells, and binding was completely blocked by CD48 monoclonal antibodies (mAbs). Using surface plasmon resonance, we showed that purified soluble mCD48 bound m2B4 with a six- to ninefold higher affinity ($K_d < 16 \mu M$ at $37^\circ C$) than its other ligand, CD2. Human CD48 bound human 2B4 with a similar affinity ($K_d < 8 \mu M$). The finding of an additional ligand for CD48 provides an explanation for distinct functional effects observed on perturbing CD2 and CD48 with mAbs or by genetic manipulation.

Key words: 2B4 • CD48 • natural killer cell • T cell • ligand

The majority of leukocyte surface proteins can be grouped according to the type of domain they contain (1). The immunoglobulin superfamily (IgSF) domain is the most highly represented domain type, found in 34% of leukocyte surface proteins. Of these, 45% have extracellular regions comprised of two IgSF domains in tandem, an arrangement that appears to be particularly favored for cell–cell recognition molecules, including the TCR. Other two-IgSF-domain leukocyte surface antigens known to mediate cell–cell interactions include CD2, CD58, CD48, CD80, CD86, CD102 (1), and OX2 (2). Among these, CD2 and its ligands, CD58 in humans (3, 4) and CD48 in rodents (5, 6) belong to the CD2 subset of the IgSF, which also includes 2B4, CDw150, CD84, and Ly-9 (Fig. 1), all of which are expressed on hematopoietic cells (1, 7, 8). With the exception of Ly-9, the extracellular regions of all of these molecules contain a membrane distal IgSF V-set domain and a membrane proximal C2-set domain (Fig. 1A). In Ly-9, this arrangement is duplicated to give four IgSF domains. The related sequence and chromosomal location of this subset together with interactions among members suggest they evolved from one molecule which mediated homophilic recognition (1, 9).

The 2B4 antigen was identified as an antigen on NK cells and T cells capable of non-MHC–restricted killing (10). 2B4 is also expressed on murine dendritic epidermal γ/δ T cells (DETCs [11, 12]). A correlation in DETCs between 2B4 expression and capacity to lyse transformed keratinocytes suggests a role for 2B4 in killing skin tumors (11). Evidence for a role in regulation of NK and T cell activity by 2B4 is provided by the cross-linking effects of the 2B4 mAb, which enhances lytic function of 2B4-expressing cells, suggesting that 2B4 can transduce stimulatory signals (10, 12). Costimulation experiments with CD3 and 2B4 mAbs suggest 2B4, like CD2 (7), can enhance signals through the TCR (12). However, the mechanisms by which 2B4 and CD2 regulate cell activation are presumably different, as there are marked differences in their cytoplasmic regions. For example, the 2B4 cytoplasmic region contains tyrosine motifs that are potential targets for phosphorylation (13), whereas the CD2 cytoplasmic region contains proline-rich motifs and no tyrosine residues (14).

Since CD2 binds to CD58 and CD48, it seemed reasonable to predict that 2B4 would also bind another molecule within the CD2 subfamily. Interactions between leukocyte...
Materials and Methods

mAbs. mAbs used were 2B4 (10); rat (r)CD4d3+4 mAb, OX68; mouse (m)CD2 mAb, RM 2.1 (19); mCD48 mAb, OX 78; rCD4 mAb, OX 45; and rCD 147 mAb, OX 47. The OX mAbs are all referenced in the European Collection of Animal Cell Cultures (Porton Down, Wiltshire, UK). Purified H M 48-1 (hamster anti–mouse CD48 [5]) was provided by Dr. H. Yagita (Juntendo University School of Medicine, Tokyo, Japan), and control 2C11 (hamster anti–mouse CD3) by Dr. E. Adams (Sir William Dunn School of Pathology). Human (h)CD48 mAbs were MEM 102 (20) and 6.28 (21), provided by Drs. V. Horejsi (Institute of Moleculcule Genetics, Prague, Czech Republic) and D. M. Thorley-Lawson (Department of Pathology, Tufts University, Boston, MA), respectively.

Vector Construction for Recombinant Proteins. The constructs for CD4d3+4 chimeric proteins used previously (22) were modified to contain in the COOH terminus a consensus peptide sequence recognized by the Esherichia coli biotin hololysenzyme synthetase, BirA (17, 18). DNA encoding the amino acid sequence NSGSL-HHILDAQKMVWNHR* was amplified by PCR using 5′ (agggttgattcctgaactcatataatcgg) and 3′ (ctcattgatctcctagcatgaccc) oligonucleotides (restriction enzyme sites are underlined) and an HLA A2 construct as template (17), provided by Dr. Paul Moss (Institute of Molecular Medicine, Oxford, U.K.). This fragment was inserted via a 5′ EcoRI site and a 3′ BamHI site into a modified Bluescript (BS KS+) vector containing a rat CD4 leader and rat CD4d3+4 (CD4Ld3+4R1 [23]). An XbaI-BamHI fragment encoding XbaI-CD4L-Sall-CD4d3+4-EcorI-biotin peptide-stop-BamHI was transferred to a modified EcoRI-biotin peptide-stop-BamHI was transferred to a modified pEF-BOS vector (24), pEF-BOS-XB. To construct fusion proteins was used to attach the recombinant molecules lineled) and an HLA A2 construct as template (17), provided by Dr. Paul Moss (Institute of Molecular Medicine, Oxford, UK). This fragment was inserted via a 5′ EcoRI site and a 3′ BamHI site into a modified Bluescript (BS KS+) vector containing a rat CD4 leader and rat CD4d3+4 (CD4Ld3+4R1 [23]). An XbaI-BamHI fragment encoding XbaI-CD4L-Sall-CD4d3+4-EcorI-biotin peptide-stop-BamHI was transferred to a modified pEF-BOS vector (24), pEF-BOS-XB. To construct fusion proteins was used to attach the recombinant molecules.
formed as described (26). Increasing and decreasing concentrations of mCD48 (5-μl injections at 20 μl/min) were passed over m2B4-CD4d3 +4, rCD5-CD4d3 +4, and mCD2. Kinetic measurements were made at 37°C (28). mCD48 (23.4 μM) was injected for 3 s at 100 μl/min over high and low levels of m2B4-CD4d3 +4, rCD5-CD4d3 +4, and mCD2. Similarly, to measure affinity, hCD48 was passed over h2B4-CD4d3 +4 and CD4d3 +4, and for kinetics, hCD48 (14.4 μM) was passed over high and low levels of h2B4-CD4d3 +4 and CD4d3 +4. Dissociation rates were measured for mCD48 as described (28) using Origin software, version 5.0 (Microcal Software, Inc., Northampton, MA). The effect of 2B4 mAb on mCD48 (23.4 μM) binding was tested by first passing 2B4 mAb at 10 μg/ml at 5 μl/min over m2B4-CD4d3 +4 until saturation was reached.

Results

m2B4 Binds to CD48. A binding reagent for a m2B4 ligand was constructed by expressing the two IgSF domains of 2B4 as a recombinant chimeric protein with domains 3 and 4 of rCD4 (m2B4-CD4d3 +4; Fig. 1B). The recombinant protein differed from previously described CD4d3 +4 chimeras (22) in that it contained a peptide sequence at its COOH terminus that can be specifically biotinylated by the *E. coli* enzyme, BirA (18). The expressed protein was biotinylated by the BirA enzyme in vitro and tested for reactivity with streptavidin and 2B4 mAb by surface plasmon resonance (not shown). The in vitro biotinylation of the recombinant protein simplified the previously described method (16) for detecting low-affinity interactions at the cell surface by enabling the biotinylated m2B4-CD4d3 +4 to be directly immobilized on avidin-coated beads (Fig. 1C).

![Figure 1. CD2 IgSF subfamily.](image)

Figure 1. CD2 IgSF subfamily. (A) Schematic representation of mouse CD2, CD48, 2B4, and Ly-9 and human CD58, CDw150 (SLAM), and CD84. (B) Recombinant chimeric m2B4-CD4d3 +4 protein. (C) The 2B4-CD4d3 +4 fluorescent beads used for ligand identification.

![Figure 2. m2B4-CD4d3 +4 fluorescent beads bind to rodent CD48 on cells.](image)

Figure 2. m2B4-CD4d3 +4 fluorescent beads bind to rodent CD48 on cells. m2B4-CD4d +3 +4 fluorescent beads bind to LPS (A and D) and Con A (B)–activated and resting (C) mouse spleen cells and rat mast cell line (Fig. 2E). This binding was completely inhibited by a rat CD48 mAb without the use of a CD4 d3 +4 mAb. The assay was shown to be at least as sensitive as coupling CD4d3 +4 fusion proteins to beads using a CD4 d3 +4 mAb (data not shown).

m2B4-CD4d3 +4 beads bound to LPS and Con A–activated mouse spleen cells (Fig. 2, A and B). The broad distribution of the ligand and our own expectation that it might be another member of the CD2 subset suggested CD48 as a likely candidate. In support of this, the binding of m2B4-CD4d3 +4 beads was blocked by the CD48 mAb, OX78, but not by a control CD2 mAb (Fig. 2, A and B). A second CD48 mAb, HM 48-1, which blocks the CD2–CD48 interaction (5) and is known to have functional effects in vivo (29, 30), also inhibited the binding of m2B4-CD4d3 +4 beads (Fig. 2C).

Preincubation of m2B4-CD4d3 +4 beads with 2B4 mAb before presentation to cells partially inhibited binding, whereas a control CD4d3 +4 mAb had no effect (Fig. 2D). The partial inhibition suggests that sites recognized by 2B4 mAb and CD48 may be close but do not overlap.

The m2B4-CD4d3 +4 beads also bound to rat thymocytes (not shown) and a rat mast cell line (Fig. 2E). This binding was completely inhibited by a rat CD48 mAb.
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(Fig. 2 E), indicating that the 2B4–CD48 interaction is conserved between these species, as is the CD2–CD48 interaction (27).

h2B4 Binds to CD48. The finding that rodent 2B4 and CD48 bind to each other suggests that 2B4 may be a ligand for CD48 in humans. The recent isolation of h2B4 cDNA (Boles, K., and P.A. Mathew, manuscript in preparation) made it possible to test this hypothesis. An h2B4 rCD4d3+4 fusion protein with a COOH-terminal biotinylation tag (h2B4-CD4d3+4) was expressed at levels similar to those obtained with the m2B4-CD4d3+4 construct, consistent with it being correctly folded. Biotinylated h2B4-CD4d3+4 immobilized on fluorescent beads bound to PBMCs (Fig. 3 A). Two hCD48 mAbs (6.28 and MEM 102) were tested for blocking effects on h2B4-CD4d3+4 cell binding. Preincubation of cells with the mAbs revealed blocking of h2B4-CD4d3+4 binding by 6.28 mAb but not by MEM 102 mAb (Fig. 3 A). Both 6.28 and MEM 102 mAbs bound to the cells, with the IgG3 6.28 mAb giving a lower signal as detected by the FITC-labeled secondary reagent (Fig. 3 B). The specificity of blocking by a CD48 mAb against a particular epitope provides convincing evidence that antigenically active h2B4 is binding to CD48 at the cell surface.

Affinity and Kinetic Analysis of the 2B4–CD48 Interaction. The finding that mCD48 has two ligands, 2B4 and CD2, raises the question of which interaction dominates in a particular response. This will depend on the distribution and level of expression of the proteins and the affinities of the two interactions. The latter was investigated by analysis on a BIAcoreTM, which permits protein interactions to be followed in real time using a detection system based on

![Figure 3](image)

**Figure 3.** h2B4-CD4d3+4 fluorescent beads bind to hCD48 on cells. (A) h2B4-CD4d3+4 fluorescent beads bind to PBMCs. Preincubation of cells with hCD48 mAb 6.28 but not MEM 102 blocked binding to the level seen with the negative control, rCD4d3+4. (B) C48 mAbs 6.28 and MEM 102 bind to the cells in A as detected with a fluorescent-labeled second mAb.

![Figure 4](image)

**Figure 4.** Affinity and dissociation rate of soluble mCD48 binding to m2B4-CD4d3+4. (A) Soluble mCD48 was injected at the indicated concentration through flow cells with immobilized m2B4-CD4d3+4 (3,733 RU) or as a negative control, rCD5-CD4d3+4 (1,857 RU), at 37°C. (B) The difference between the response at equilibrium in the m2B4-CD4d3+4 and control flow cells is plotted against the mCD48 concentration. A $K_{d} = 15 \mu$M and maximum binding of 1,197–1,225 RU were calculated by nonlinear curve fitting of the Langmuir binding isotherm (line) to data (circles) from A with negative control subtracted. (C) Scatchard plot of data in B plus data for mCD48 binding mCD2. (D) Soluble mCD48 (23.4 $\mu$M) was injected over immobilized m2B4-CD4d3+4 at high (1,060 RU) and low (572 RU) levels, mCD2 (557 RU), and rCD5-CD4d3+4 (1,083 RU). A $k_{d} = 3 s^{-1}$ for 2B4, 22 $s^{-1}$ for mCD2, and 41 $s^{-1}$ for rCD5-CD4d3+4 was calculated by exponential decay curve fitting (line) to dissociation data (symbols). (E) In the experiment shown in D, soluble mCD48 (23.4 $\mu$M) was injected over immobilized m2B4-CD4d3+4 (1,060 RU) before and after saturation with 2B4 mAb.
surface plasmon resonance. The affinity was measured by determining the level of binding at equilibrium after injection of a range of concentrations of mCD48 over immobilized m2B4-CD4d3+4 and mCD2. Immobilized rCD5-CD4d3+4 was used as a negative control in the reference flow cell. A representative experiment with increasing concentrations of mCD48 injected at 37°C is shown in Fig. 4 A. The difference between the response at equilibrium in the m2B4 and control flow cells represents binding (Fig. 4 B). A Scatchard transformation of the data is shown in Fig. 4 C. mCD48 bound to m2B4 with a $K_d = 16$ μM at 37°C (Fig. 4, B and C, and Table 1). This was significantly higher than the mCD48-mCD2 interaction ($K_d = 90$ μM) measured simultaneously (Fig. 4 C, and Table 1). Values determined for the mCD48-mCD2 interaction agree with published data (27; Table 1). At 25°C, mCD48 bound m2B4 and mCD2 with a $K_d$ of 7 and 49 μM, respectively (data not shown).

Kinetic studies were carried out at 37°C to determine the dissociation rate constant ($k_{off}$) of the mCD48-m2B4 interaction. mCD48 dissociated more slowly from m2B4 ($k_{off} = 3$ s$^{-1}$) than from mCD2 ($k_{off} > 20$ s$^{-1}$) (Fig. 4 D). The $k_{off}$ was the same when the level of immobilized m2B4-CD4d3+4 was decreased, indicating that the dissociation rate measured was not affected by rebinding or mass transport limitations. This suggests that the higher affinity of the 2B4-CD48 interaction is the result of a decreased $k_{off}$.

The partial blocking effect of 2B4 mAb on cell binding (Fig. 2 D) was reproduced in Biacore™ analysis as shown in Fig. 4 E. Saturation of m2B4-CD4d3+4 with 2B4 mAb before injection of mCD48 reduced the level of mCD48 binding.

To measure the affinity of the interaction between h2B4 and hCD48, a soluble form of hCD48 was produced. In analyses similar to that carried out for the m2B4-mCD48 interaction, affinity measurements were made with soluble hCD48 and immobilized h2B4-CD4d3+4. Both nonlinear curve fitting (Fig. 5 B) and linear regression analysis of the Scatchard plot (Fig. 5 C) yielded a $K_d = 8$ μM (Table 1). The apparent $k_{off}$ was 5 and 7 s$^{-1}$ when measured with high and low levels of immobilized h2B4 (Fig. 5 D). This indicates that dissociation is limited by mass transport and/or rebinding and that the true $k_{off}$ is $> 7$ s$^{-1}$.

Table 1. Comparison of Affinity and Dissociation Rates for Soluble CD48 Binding to Immobilized 2B4 and CD2 at 37°C

| Immobilized | Soluble  | $K_d$  | $k_{off}$ |
|-------------|----------|--------|----------|
| m2B4        | mCD48    | 16     | 3        |
| h2B4        | hCD48    | 8      | $> 7$    |
| mCD2        | mCD48    | 90*    | $> 10^4$ |
| hCD2        | hCD58    | 9–22‡  | $> 4^*$  |

* Data from reference 27.
‡ Data from reference 28.

Discussion

The interaction between 2B4 and CD48 identified in this study represents a new ligand pair that is conserved between rodents and humans within the CD2 subset of IgSF molecules. A previous study failed to demonstrate an additional ligand for CD48 in the rat (16). However, 2B4 is expressed on a limited range of cell types, and these were not examined (16). Interestingly, in preliminary experiments, the binding of biotinylated multivalent CD48-CD4d3+4 to lymphokine-activated killer (LAK) cells shown to express both 2B4 and CD2 was entirely blocked by a CD2 mAb (Brown, M.H., unpublished observations). It is possible that with the particular conditions used to generate the LAK cells, too few expressed 2B4 at a sufficiently high surface density and/or 2B4 has insufficient lateral mobility to support multivalent binding. Limitations of this type of assay have been demonstrated previously (16). Thus, our observation that CD48 mAbs completely block 2B4-CD4d3+4 binding to cells does not rule out the existence of another ligand for 2B4. Ianelli et al. have reported that an hCD48-IgM fusion protein binds to epithelial cells, which do not express CD2 (31). It is unclear whether 2B4 is the CD48 ligand on these cells.
Our finding that the m2B4–mCD48 interaction in the mouse has a significantly higher affinity than the mouse or rat CD2–CD48 interactions (26, 27; Table 1) suggests that it is biologically significant. Moreover, the strength of the 2B4–CD48 interaction is conserved across species, with similar affinities being observed for the mouse and human 2B4–CD48 interactions (27, 28). An interaction has been reported between hCD48 and hCD2 (32, 33), but this appears to be exceptionally weak (Kd > 0.5 mM [28]). Since CD2 binds with a much higher affinity to CD58 and essentially all cells that express CD48 also express CD58, it is doubtful that the CD2–CD48 interaction is of physiological significance in humans. In contrast, the relatively high affinity of h2B4 for hCD48 suggests that this latter interaction is physiologically important. The same CD48 mAbs block CD2 and 2B4 binding to CD48, suggesting that the 2B4 ligand binding site, like the CD2 binding site (34), lies on the GFCC ‘C’ face of the V domain. Thus, a similar topology for the CD2–CD48 and 2B4–CD48 complexes can be predicted. The dimensions of the CD2–CD48 complex are predicted to be similar to the dimensions of the TCR–MHC complex (14 nm from membrane to membrane [7]). This has led to the suggestion that the maintenance of this intercellular distance is important for initiation of TCR–peptide MHC ligation (7). Interestingly, structure determination of an N K killer inhibitory receptor (KIR [35]) predicts that when bound to MHC class I, the complex will also span a similar distance. Together with functional studies (for a review, see reference 36), this suggests that CD2 on NK cells may contribute to activation through NK receptors. This hypothesis is supported by studies with mAbs and CD58-transfected cells that suggest that CD2 ligation can enhance activation of NK cells (36, 37). By analogy with CD2 (7) and substantiated by experiments with 2B4 mAb (10, 12), the 2B4–CD48 interaction may enhance NK and TCR ligand interaction as well as contributing to signals initiated through NK receptors and the TCR.

The expression on the same lymphocytes of two proteins (2B4 and CD2) that bind the same ligand (CD48) raises the question of whether CD2 and 2B4 have distinct functions. An analogy is exemplified by the well-characterized interactions of the T cell molecules CD28 and CD152, which bind the same ligands, CD80 and CD86 (38). There are several interesting parallels that can be drawn between these two pairs of molecules (CD28/CD152 and 2B4/CD2) that share ligands. First, they have different affinities for the same ligand, i.e., 2B4 and CD2 have different affinities for CD48, and CD152 has a 10-fold higher affinity than CD28 for CD80 (39). Second, their patterns of expression differ. The expression of both 2B4 and CD152 is much enhanced on activated cells (10, 11, 38), whereas both CD2 and CD28 are present on resting and activated cells. Third, they have different cytoplasmic regions. In the case of CD28 and CD152, this results in completely different functions, with CD28 stimulating and CD152 inhibiting (38). Thus, it seems likely that the cytoplasmic portions of 2B4 and CD2 will confer different functions. The more restricted expression of 2B4 on cells associated with non-MHC-restricted killing compared with CD2, which is present on virtually all T cells, suggests the primary role of 2B4 may be in generation of lytic activity. This is supported by the absence of effects of CD48 mAbs on target recognition (29).

Experiments have been done in vitro and in vivo with CD48 and CD2 mAbs that can now be reinterpreted in the light of there being two ligands for mCD48. For example, a CD48 mAb (HM 48-1) has been shown to inhibit development of non-MHC-restricted lytic activity, whereas a blocking CD2 mAb had no effect (29). One explanation for this is that it is the 2B4–CD48 and not the CD2–CD48 interaction that has a major role in the development of the lytic population. In vivo, the synergistic effects of blocking CD2 and CD48 mAbs on allograft survival may be explained by disruption of the 2B4–CD48 and the CD2–CD48 interactions (30). Clearly, further experiments are needed to clarify this point. The activating properties (10) and partial inhibition of the 2B4–CD48 interaction make the currently available 2B4 mAb unsuitable for such experiments. Finally, recent observations that there are differences in phenotype between CD48- (40) and CD2-deficient mice (41, 42) can now be investigated with reference to the existence of an alternative ligand for CD48.
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