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Ligation of Human CD46 with Purified Complement C3b or F(ab')$_2$ of Monoclonal Antibodies Enhances Isoform-Specific Interferon Gamma–Dependent Nitric Oxide Production in Macrophages[1]

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CD46, a complement regulatory protein widely expressed on human cells, serves as an entry receptor for measles virus (MV). We have previously shown that the expression of human CD46 in mouse macrophages restricts MV replication in these cells and enhances the production of nitric oxide (NO) in the presence of gamma interferon (IFN-γ). In this study, we show that crosslinking human CD46 expressed on the mouse macrophage-like cell line RAW264.7 with purified C3b multimer but not monomer enhances NO production. The enhanced production of NO in response to IFN-γ was observed again with C3b multimer but not monomer. The augmentation of NO production is human CD46-dependent with a CYT1>CYT2 profile. Thus, the reported MV-mediated NO production, irrespective of whether it is IFN-γ–dependent or –independent, should be largely attributable to CD46 signaling but not to MV replication. Similar CYT1-dependent augmentation of NO production was reproducible with two CD46 ligating reagents, CD46-specific monoclonal antibodies (mAb) or their F(ab')$_2$ and MV hemagglutinin (H) and fusion (F) glycoproteins. Co-cultivation of mouse macrophages bearing human CD46 with Chinese hamster ovary (CHO) cells expressing MV H and F enhanced IFN-γ–induced NO production. Yet, the NO levels induced by F(ab')$_2$ against CD46 or MV H/F on CHO cells were much lower than those induced by CD46-crosslinking mAb with Fc or MV infection. Removing the cytoplasmic tails of CD46 abrogated the augmentation of NO production triggered by all three stimulators. Thus, the CD46 CYT1 and CYT2 isoforms functionally diverge to elicit innate immune responses, which can be modulated by purified C3b multimer or anti-CD46 mAbs.

Key words: CD46 (membrane cofactor protein), complement regulator/receptor, measles virus receptor, nitric oxide induction, macrophages.

Membrane cofactor protein (MCP or CD46) is an integral complement regulatory protein widely expressed on nucleated human cells/cell lines. Its best known function is to protect host cells from complement-dependent cytolysis (CDCC) by inactivating C3b and C4b together with protease factor I and regulating membrane deposition of these effector molecules (*1, 2*). The extracellular portion of CD46 consists of a series of short consensus repeats (SCR1, SCR2, SCR3, and SCR4) responsible for binding to C3b/C4b. These domains are followed by regions rich in serine and threonine (ST-A,B,C) and a hydrophobic transmembrane region (*1, 3*). The ST portion of CD46 may control ligand binding efficacy resulting in control of CD46 function (*4*). The cytoplasmic portion of CD46 consists of a common juxtamembrane sequence and different distal sequences generated by differential mRNA splicing (*1, 3, 5*). The most common forms of the cytoplasmic domains of CD46 in human cells are called CYT1 and CYT2 (*1, 3, 5*). Other isoforms, CYT3 and CYT4, are found in testicular germ cells (*5, 6*), but not in other organs. The extracellular portion of the CD46 molecule is sufficient to protect cells against complement-mediated lysis (*7–10*). The requirements of a variety of cytoplasmic domains of CD46 and their functions are largely unknown.

Recent studies have suggested that in addition to the

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Abbreviations: CHO, Chinese hamster ovary; CYT1, an isoform of human CD46; CYT2, an isoform of human CD46; DACM, dimethylamino-4-(methylcoumarinyl)maleimide; DC, dendritic cells; F, fusion protein; H, hemagglutinin protein; IFN-γ, gamma interferon; mAb, monoclonal antibodies; MCP, membrane cofactor protein (CD46); MV, measles virus; NO, nitric oxide; SCR, short consensus repeat.

known complement regulatory function, CD46 may have signaling functions that modulate cellular responses. In human B cells, crosslinking of CD46 synergizes with IL-4 to enhance IgE class switching (*11*). In human T cells, CD46 may serve as a co-stimulator enhancing CD3-mediated cell proliferetion (*12*). In human macrophages (*13*), crosslinking CD46 with MV, dimerized C3b or CD46-specific mAbs leads to the inhibition of IL-12 production in response to LPS or *Staphylococcus aureus* cowan (SAC). In human dendritic cells (*14, 15*), MV causes increased expression of co-stimulators and IL-12, but results in failure to induce lymphocyte proliferation. In other cells, crosslinking CD46 on human astrocytoma cells with CD46-specific antibody increases the production of IL-6 (*16*). CD46 also appears to serve as a receptor for pathogenic *Neisseria* (*17*). Binding of *Neisseria gonorrhoeae* to human epithelial cells causes a transient increase in intracellular calcium levels, and this response is blocked by antibody against CD46 (*18*). These lines of evidence suggest that CD46 may act as a signaling molecule that modulates cellular functions.

CD46 serves as an entry receptor for laboratory-adapted strains of measles virus (MV) (*19, 20*). Interestingly, infection by MV induces the same responses as crosslinking CD46, such as the suppression of IL-12 production from human monocytes/macrophages (*13*), induction of IL-6 in human astrocytoma cells (*16*), and enhancement of IgE class switching in a human B cell line upon IL-4 treatment (*11*). In addition, we have shown that the expression of human CD46 in mouse macrophages does not render these cells more permissive for MV, but further restricts virus replication (*21*). Mouse macrophages expressing human CD46 produce higher levels of NO upon infection by MV in the presence on IFN-γ (*21*). NO production can be modulated by type 1 IFNs in MV-infected macrophages (*22*). Although there is no direct evidence, these responses could be attributable to the CD46-mediated signaling (*23, 24*). Thus, it is important to establish a system in which to test CD46-mediated cellular responses using pure ligands with no amplifiable villions or bioactive material.

In the present study, we investigated whether ligand-mediated ligation of the CYT1 or CYT2 isoform of human CD46 expressed on mouse macrophages could enhance IFN-γ–dependent NO production using purified ligand or ligand transfectants. We conclude that purified ligands can induce CD46-mediated immune responses even under non-infectious conditions.

MATERIALS AND METHODS

Cells and Reagents—RAW264.7 mouse macrophages (a gift from Dr. Alan Aderem, University of Washington) stably expressing human CD46 with a cytoplasmic domain of CYT1 (A24), CYT2 (B24), or tail-less CD46 mutant (C11) were generated as described previously (*21*). Their mean fluorescence shifts determined by flow cytometry using M177 (*8*) were 3.64, 2.88, and 15.61, respectively. Briefly, RAW264.7 cells were transfected by electroporation with plasmids based on the pME18S vector, encoding human CD46 with CYT1, CYT2 or lacking a cytoplasmic domain, under control of the SRα promoter, and the neomycin resistance gene driven by the promoter of reticuloendotheliosis virus (REVneo^R). Control cells (F7) were transfected with the same vector without CD46 cDNA (*24*). Cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum (GIBCO BRL, Grand Island, NY) and 400 μg per ml of G418 (neomycin analogue; GIBCO BRL). For C3b stimulation experiments (Fig. 3b), we additionally provided high expression levels of CYT1 (CY1#46) and CYT2 (CY2#4), for which the mean fluorescence shifts determined by M177 were 11.33 and 10.20, respectively. Chinese hamster ovary (CHO) cells expressing MV hemagglutinin (H), fusion (F), or H and F proteins were generated by transfecting CHO cells using the calcium-phosphate method (*8*) with a pME18S-based vector encoding MV (Nagahata strain) H or F protein under the SRα promoter, together with pREV neo^R plasmid (*25*). Expression of viral proteins in G418-resistant cell clones was examined by immunoprecipitation of [^{35}S]methionine-labeled cell lysates with monospecific antibodies against the MV H or F protein (*26*). These cells were maintained in Eagle's MEM supplemented with 10% newborn calf serum (GIBCO BRL) containing 400 μg per ml of G418.

Recombinant murine IFN-γ, normal mouse IgG2a, and the F(ab′)$_2$ fragment of goat anti–mouse IgG were purchased from Pharmingen (San Diego, CA) and Pierce (Rockford, IL), respectively. Affinity-purified IgG and F(ab′)$_2$ fragments of anti-CD46 mAb, M160 (IgG2a), and M177 (IgG1) were described previously (*27, 28*). The F(ab′)$_2$ fragments were purified on an immobilized protein A column and had no detectable Fc receptor–binding activity (*28*).

Expression of FLAG-Tagged CYT1 and CYT2 of CD46—Cells stably expressed CYT1 (CY1#46) or CYT2 (CY2#4) were transfected with FLAG-tagged CYT2 or FLAG-tagged CYT1, respectively. The tagged constructs were made as previously reported (*29*). Briefly, the CYT1 and CYT2 cDNAs with the region of the signal sequence removed were inserted at the *Not*I/*Not*I site of pFLAG-CMV#1 expression vector (Sigma, St. Louis, MO). The 5′ *Not*I site was attached to the nucleotide sequence corresponding to the N-terminal portion of the CYT1/CYT2 cDNAs. The other *Not*I site was placed adjacent to the TAG stop codon. That is, the final constructs consisted of the pFLAG-CMV#1 signal peptide sequence followed by the FLAG sequence, *Not*I site (DDDDKLAAA, gacgatgacgacaagcttgcggccgc), the regions of the protein sequences of CYT1 and CYT2, and the *Not*I site. The final sequences were confirmed by DNA sequencing. CY1#46 cells or CY2#4 cells (1×10^7) were transfected with 5 μg of the plasmids by the LipofectAMINE method. Forty-eight clones were randomly harvested and cultured in a 24-well plate. The ratios of CYT1 to CYT2 on the CY1#46 or CY2#4 clones were evaluated by flow cytometry using anti-FLAG antibody, and clones with various expression ratios of CYT1/CYT2 were established. These cells were used for the NO production assay (see below).

Protein Analysis—Cells (1×10^7) were lysed in 200 μl of lysis buffer (1% NP-40, 10 mM EDTA, 25 mM IAA, 2 mM PMSF, DPBS) for 20 min at room temperature. After centrifugation at 100 ×*g* for 10 min, the supernatant was centrifuged again at 200,000 ×*g* for 1 h at 4°C. Aliquots of 50 μl of the supernatant were subjected to SDS-PAGE (10% gel) under non-reducing or reducing conditions. After electrophoresis, the resolved proteins were transferred onto nitrocellulose sheets. The sheets were then blocked with 10% skimmed milk for 1 h at 37°C, then overnight at 4°C, and sequentially incubated with mAb (10 μg/10 ml PBS con-

taining 0.02% NP-40) and 1 μg/10 ml of HRP-conjugated goat anti–mouse IgG (Bio-Rad), followed by staining with an ECL kit (Amersham Pharmacia Biotech).

Biosynthetic Labeling—The expression of MV glycoproteins in CHO cells was determined by labeling the cells with [^{35}S]methionine (New England Nuclear Life Sciences, Boston, MA) for 1 h and immunoprecipitating the cell lysates with MV H mAb (*26*), or monospecific rabbit antiserum against the MV F protein (*26*).

Preparation of C3b and C3b Multimer—Human C3 was purified from human serum as described previously (*27*). Methylamine-treated DACM-labeled C3b (DACM-C3b) was prepared in our laboratory (*27*). The C3b multimer was prepared using a linker DTSSB (Pearse) and dithiothreitol, and separated from the monomer by gel filtration as described previously (*30*). The purity of the multimer was checked by SDS-PAGE followed by dye staining and immunoblotting using mAbs against human C3b and FITC-labeled second Ab (*27*).

Flow Cytometry—Cells (1×10^6) suspended in 50 μl of DPBS containing 0.5% BSA and 0.1% NaN_3 (BSA/NaN_3/DPBS) were mixed with 50 μl of EDTA-plasma and 5 μg of mouse IgG or mAb, and incubated for 30 min at 4°C. After washing with BSA/NaN_3/DPBS, the cells were suspended in 90 μl of BSA/NaN_3/DPBS and incubated with FITC-labeled goat F(ab')$_2$ of anti–mouse IgG at 4°C. After 30 min, the cells were washed twice with DPBS and fixed with paraformaldehyde. The samples were analyzed on a FACS Calibur (Becton-Dickinson).

Nitrite (NO_2^-) Determination—RAW cells (2.5×10^5) were treated with C3b (10–100 μg/ml) for 1 h at 4°C and further incubated for 24 h with or without 200 U/ml of mouse IFN-γ. In other experiments, RAW cells (2×10^6/well) were mixed with mAbs (10 μg/ml) and allowed to stand for 1 h at 4°C. Then, 800 μl of warmed medium or medium containing IFN-γ (final concentration 200 U/ml) was added and the cells were cultured at 37°C for 24 or 48 h. Culture media were collected in either case. MV H/F-expressing CHO cells (2×10^6) were incubated in the presence (+) or absence (–) of 100 U per ml of IFN-γ. Twenty-four hours after co-cultivation, the culture medium was collected. NO production was examined by determining the concentrations of stable end product NO_2^- in the culture medium by a colorimetric method (*21, 31*). Triplicate samples containing equal volumes (100 μl) of cell-free culture medium and Greiss reagent (1% sulfanilamide, 0.1% *N*-1-naphthylenediamine, 5% H_3PO_4) (Sigma Chemical) were mixed in 96-well plates at room temperature for 15 min. Optical density was measured with a microplate reader (EL808; Bio Tek Instrument) at 550 nm. A standard curve was generated for each experiment using known concentrations of sodium nitrite as references.

RESULTS

CD46 Isoform–Specific Divergent Regulation of IFN-γ–Induced NO Production—To test directly whether interaction with human CD46 on the surface of mouse macrophage lines enhances NO production, we treated mouse macrophage lines F7 (CD46-negative), A24 (CD46 with CYT1 domain), B24 (CD46 with CYT2 domain), and C11 (CD46 without cytoplasmic domains) with M160 mAb against the SCR3 domain of human CD46, in the absence or presence of IFN-γ. Control cultures were treated with medium or an equivalent amount of isotype-matched control mAb (IgG2a). NO production was monitored the following day by measuring NO_2^- concentrations. The A24, B24, and C11 mouse macrophage cell lines expressed comparable levels of human CD46 with or without cytoplasmic tails (data not shown). In the absence of IFN-γ, treating CD46-negative (F7) or CD46-positive mouse macrophage lines (A24, B24, C11) with CD46-specific or control mAb had no significant effect on NO production (Fig. 1, lanes a–l). We previously determined that 300 U/ml of IFN-γ is optimum for maximal differentiation of the NO response between CD46-positive *versus* CD46-negative mouse macrophages (*21*). Exposure to 300 U/ml of IFN-γ induced various levels of NO production in all mouse macrophage lines examined (Fig. 1, lanes m–x). Treatment with M160 mAb further enhanced NO production in the A24 macrophage line expressing human CD46 with the CYT1 cytoplasmic tail (Fig. 1, lane r). In contrast, NO production in the B24 macrophage line with the CYT2 isoform was slightly suppressed by M160 mAb under the same conditions (Fig. 1, lane u). This mAb did not increase NO production in F7 macrophages lacking CD46 (Fig. 1, lanes m–o). Isotype-matched mAb failed to significantly influence NO production in these macrophage cell lines (Fig. 1, lanes n, q, t, and w) compared to those without antibody treatment (Fig. 1, lanes m, p, s, and v). Most importantly, M160 mAb failed to enhance IFN-γ–induced NO production in C11 macrophages expressing the extracellular domains of CD46 without a cytoplasmic domain (Fig. 1, lanes v–x).

These results indicate that the binding of CD46-specific mAb to the CYT1 isoform of human CD46 expressed on mouse macrophages enhances NO production in response to IFN-γ. This augmenting effect is specific, since control mAb did not enhance NO production. Furthermore, the CYT2 isoform tended to suppress IFN-γ–dependent NO production. The cytoplasmic domains of CD46 are crucial

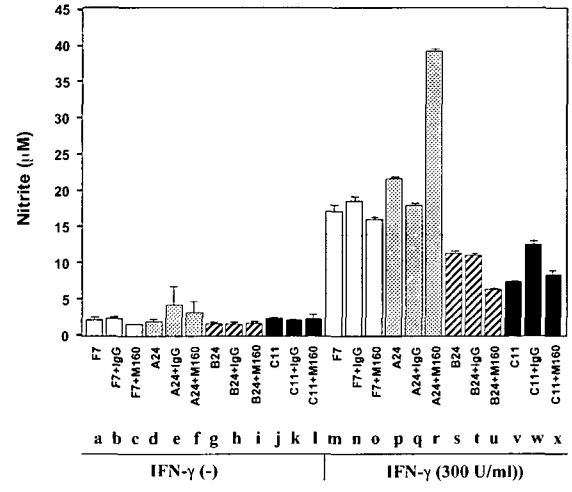


Fig. 1. **NO production from mouse macrophages crosslinked with CD46-specific antibody.** Mouse macrophages F7, A24, B24 or C11 (2×10^6) were incubated in medium with no antibody, 10 μg per ml of CD46-specific M160 mAb, or isotype-matched control mouse IgG in the presence (300 U per ml) (lanes m–x) or absence (–) (lanes a–l) of IFN-γ. Nitrite in the culture medium was assayed 24 h later. Bars atop each column represent standard deviations determined from 3 independent measurements.

for this dual modulating feature of NO production upon the binding of antibody to CD46 extracellular domains.

F(ab')$_2$ but Not Fc Governs CYT1 CD46-Mediated Enhancement of NO Production—In order to rule out definitively the involvement of Fc receptors in the observed NO response, we further examined NO production in IFN-γ–treated A24 and C11 mouse macrophages by specifically crosslinking CD46 with purified F(ab')$_2$ of M160 mAb followed by F(ab')$_2$ of secondary goat anti–mouse IgG. No Fc fragment was detected by silver-staining on SDS-PAGE, indicating that the level of contaminating Fc fragments was below the limit of detection (<0.5 ng/10 μg) in the F(ab')$_2$ preparation (data not shown). As shown in Fig. 2, crosslinking CD46 with F(ab')$_2$ of M160 mAb also significantly enhanced NO production in A24 mouse macrophages on days 2 and 3, compared to a parallel culture treated with isotype-matched IgG2a and F(ab')$_2$ of secondary goat anti–mouse IgG (Fig. 2, lanes a, b, g, and h). Furthermore, crosslinking the tail-less CD46 mutant on C11 macrophages with the same amount of F(ab')$_2$ of M160 mAb and secondary F(ab')$_2$ did not enhance NO production on day 2 after treatment (Fig. 2, compare lanes e and f). On day 3 after crosslinking the tail-less CD46 mutant, the C11 culture released moderate levels of NO (Fig. 2, compare lanes k and l). Hence, CD46-specific mAb F(ab')$_2$ of M160 and M177 (data not shown) consistently elicited higher levels of NO production in the presence of IFN-γ in A24 mouse macrophages expressing human CD46 than in F7 or C11 cells lacking human CD46 or expressing a tail-less CD46 mutant. The suppressive effect of CYT2 was again observed by stimulation with F(ab')$_2$ fragment (data not shown). These results suggest that CYT1 and CYT2 CD46 modulate macrophage activation by different mechanisms. As shown in human systems (*24*) and NO production pathways (*31*), human CD46 should transmit signals from the mouse macrophage surface to augment NO production in response to IFN-γ. This response does not require co-ligation of Fc receptors.

Enhancement of IFN-γ–Dependent NO Production by Human C3b Multimer—We next examined whether the natural ligand of human CD46 up-regulates NO production in CYT1-expressing cells, A24. There has been only one previous report suggesting the participation of C3b in macrophage activation, in which the induction of IL-12 p40 by C3b dimer (*13*) was demonstrated. C3b monomer and multimers were separated by molecular sieve HPLC (Fig. 3a).

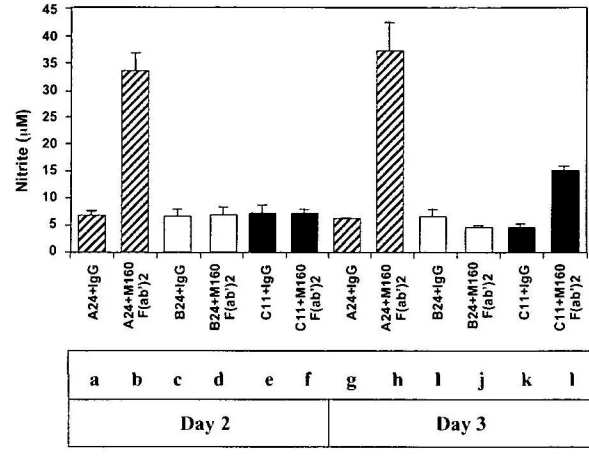
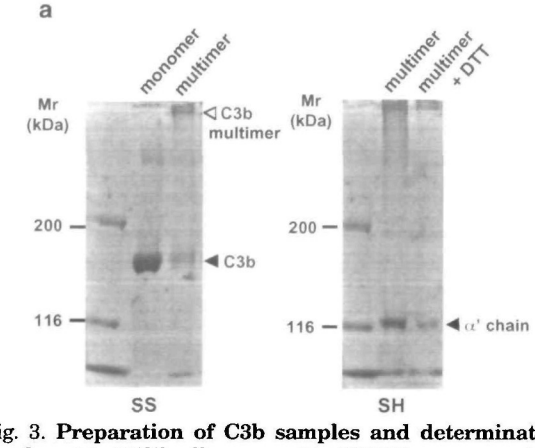


Fig. 2. **NO production from mouse macrophages crosslinked with the F(ab')$_2$ fragment of CD46-specific antibody.** Mouse macrophages F7, A24, B24, or C11 (2×10^6) were incubated at 4°C for 1 h in 200 μl of RPMI1640 medium containing 10 μg per ml of M160 F(ab')$_2$ or isotype-matched mouse IgG. Eight hundred microliters of warmed medium containing IFN-γ (final concentration 300 U/ml) and F(ab')$_2$ fragment of goat anti–mouse IgG (final concentration 10 μg/ml) was added and cells were cultured at 37°C for 24 (Day 2) or 48 h (Day 3). NO production was determined by measuring nitrite in the culture medium. Bars atop each column represent standard deviations of 3 independent measurements.



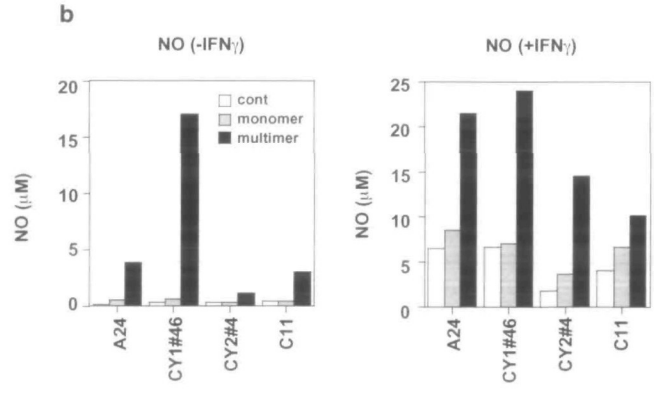


Fig. 3. **Preparation of C3b samples and determination of NO produced in RAW cells expressing CD46 in response to C3b samples.** Panel a: SDS-PAGE analysis of purified C3b monomer and multimer. Samples were prepared as described in "MATERIALS AND METHODS," and analyzed by SDS-PAGE followed by staining with Coomassie Brilliant Blue. SS, non-reducing conditions; SH, reducing conditions. Open arrowhead, C3b multimer; closed arrowhead, C3b monomer. In the right panel, the reduced alpha chain is indicated by the arrowhead. Panel b: NO production from mouse macrophages by crosslinking with C3b multimer or monomer. Mouse macrophages A24 (with low CYT1 isoform), CY1#46 (with high CYT1 isoform), CY2#4 (with high CYT2 isoform), and C11 (with tail-less CD46) (2×10^6) were incubated at 4°C for 1 h in 200 μl of RPMI1640 medium containing the same amounts of C3b monomer or multimer (25 μg/ml). Eight hundred microliters of warmed medium (left panel) or medium containing IFN-γ (final concentration 200 U/ml) (right panel) was added and the cells were cultured at 37°C for 24 h. NO production was determined by measuring nitrite in the culture medium. Three experiments were performed with similar results and the results of one representative experiment are shown.

We used fractions in which >80% of the C3b moieties were multimers. NO production was induced by C3b multimer (25 μg/ml) in the absence of IFN-γ only when the cells expressed high levels of CD46 CYT1 (Fig, 3b, left panel). NO production by A24 cells in response to IFNγ (200 U/ml) was increased by C3b multimer (Fig. 3b, right panel). Notably, CY2#4 (Fig. 3b, right panel) and B24 (not shown) cells responded subtly to C3b multimer. Thus, the results of C3b multimer show some discrepancy with those of M160 mAb, which induced a slight suppression of IFN-γ–induced NO production in CYT2-expressing cells. The CYT2-mediated response may be critical depending upon the ligands employed. In either case, C3b monomer had virtually no effect on IFN-γ–induced NO production (Fig. 3b). The augmentation of NO response was observed in a C3b multimer dose-dependent manner (10–100 μg/ml) in CYT1-expressing cells, while NO production by CYT2-expressing cells was marginally increased even at a high C3b multimer concentration (data not shown). Again, little enhancement of NO production was observed under these conditions in C11 cells with no cytoplasmic tail. C3b monomer with no contaminating multimers exhibited no enhancing effect on NO production even at 200 μg/ml in A24 and CY1#46 cells.

NO Production by RAW Cells Co-Expressing CYT1 and CYT2 Isoforms—CYT1 and CYT2 isoforms of CD46 are known to be widely distributed on human tissues/organs and are simultaneously expressed on the same cells (*5, 32*). Since CYT1 and CYT2 isoforms exhibit distinct profiles of IFN-γ–induced NO production, we next examined how cells expressing both CYT1 and CYT2 at various levels respond to CD46 ligation. RAW cells and NO production were used as the cellular base and measure of output for the determination of CD46 function. We provided RAW sublines (CY1#46 and CY2#4) with FLAG-tagged CYT2 and CYT1 isoforms, and the ratios of CYT1 to CYT2 were estimated for a number of clones by flow cytometry (Table I). The levels of FLAG-tagged CYT1 and CYT2 were assessed by anti-FLAG Ab. The levels of NO produced by these clones in response to C3b multimer (25 μg/ml) or M160 mAb (10 μg/ml) were evaluated in the presence of IFN-γ (Table I). The CYT1 phenotype that efficiently induced NO appeared to be dominant compared to the CYT2 phenotype with marginal NO induction in most clones expressing both CYT1 and CYT2. However, when the level of CYT1 was ≤30% of that of CYT2, the CYT2 phenotype tended to be expressed. Thus, human macrophages, though expressing both isoforms, should effectively produce NO responding to CD46 crosslinking by C3b or other ligands.

TABLE I. **IFN-γ–induced NO production in RAW sublines co-expressing CYT1 and CYT2 isoforms.**

RAW sublines[a]	Mean fluorescence shifts		CYT1 *vs.* CYT2[b]	NO production (μM)[c]		
	FLAG	M177		Cont.	M160	C3b multimer
CY1#46						
No. 13	4.61	16.33	0.445	15.3	42.6	26.1
No. 17	9.27	22.90	1.03	14.5	43.4	26.7
No. 22	24.54	37.88	2.35	14.4	28.9	20.8
CY2#4						
No. 36	5.63	17.03	0.670	10.7	23.1	18.0
No. 40	9.00	20.23	0.983	11.6	34.5	23.1
No. 47	18.77	30.12	1.95	12.2	34.0	23.8

[a]Clones were numbered according to the levels of FLAG-tagged proteins. No. 1–24, CY1#46 subclones; No. 25–48, CY2#4 subclones. [b]The CYT1 level (mean fluorescence shift) of CY1#46 was 11.3 while the CYT2 level of CY2#4 was 10.2. The CYT2/CYT1 ratio was evaluated in each CY1#46 subclone and the CYT1/CYT2 ratio was evaluated in each CY2#4 subclone, assuming that values above basal levels reflected the levels of FLAG-labeled CD46. [c]Cont., no ligand added; M160, 10 μg/ml of mAb M160 was added; C3b multimer, 25 μg/ml of C3b multimer was added.

Recognition of MV Glycoproteins by Mouse Macrophages Bearing Human CD46—MV-infected cells express two viral glycoproteins, H and F, on the cell surface, which allow binding to CD46 and induce membrane fusion, respectively (*33*). We examined whether the expression of human CD46 isoforms allows mouse macrophages to distinguish between target cells with or without MV glycoproteins on the cell surface. We generated CHO cells stably expressing one or both of the glycoproteins of MV Nagahata strain, which selectively uses CD46 as an entry receptor (*34, 35*) and is poorly replicated in human dendritic cells (*35*) and mouse macrophages bearing human CD46 (*21*). We first examined protein expression by labeling with [^{35}S]methionine for 1 h and immunoprecipitation with monospecific antibodies against the MV H or F protein. The CHO cell clones H4, H16, and H24 express different levels of a 75–78 kDa doublet, representing differentially glycosylated forms of H protein (Fig. 4, lanes b–d). Clones F2, F4, and F21 express different levels of the MV F protein, which appeared as the uncleaved F0 precursor after brief labeling (Fig. 4, lanes e–g). Three additional clones, HF7, HF15, and HF16, exhibited different ratios of both the H and F proteins (Fig. 4, lanes h–j). We chose the H24, F21, and HF15 clones for further studies, as these lines expressed comparable levels of H, F or both H and F proteins, respectively (Fig. 4, lanes d, g, and i). Comparable levels of the mature forms of H and F proteins were also detected on the cell surface (data not

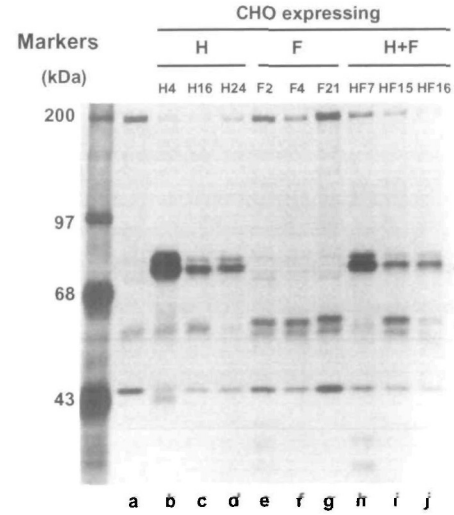


Fig. 4. **MV glycoproteins stably expressed in CHO cells.** Three independent clones of CHO cells expressing MV H protein (H4, H16, and H24; lanes b, c, and d, respectively), F protein (F2, F4, and F21; lanes e, f, and g, respectively), or H plus F proteins (HF7, HF15, and HF16; lanes h, i, and j, respectively) were labeled for 1 h with [^{35}S]methionine, along with a control macrophage clone transfected with *neoR* (lane a). The cell lysates were immunoprecipitated with H-specific mAb (lanes b–d), polyclonal antibodies specific for F (lanes e–g) or both H- and F-specific antibodies (lanes a, and h–j). The immunoprecipitated proteins were analyzed by SDS-PAGE. Protein markers are shown to the left.

shown). CHO cells transfected with the vector alone were also generated as a control (Fig. 4, lane a). Equal numbers of mouse macrophages F7, A24, and C11 were co-cultivated with CHO cells expressing different MV glycoproteins. In the absence of IFN-γ, mouse macrophages without (F7) or with human CD46 (A24, C11) produced essentially the same basal levels of NO (about 4 μM) upon co-cultivation with CHO cells expressing vector alone (Fig. 5, lanes a, i, and q). Co-cultivation with CHO cells expressing MV glycoproteins in the absence of IFN-γ did not influence NO production (Fig. 5, lanes c, e, g, k, m, o, s, u, and w). Treatment with IFN-γ increased NO production by about two-fold in F7 mouse macrophages co-cultivated with CHO cells transfected with vector alone (Fig. 5, compare lanes a and b). The expression of MV glycoproteins on CHO cells did not change the levels of NO production by F7 macrophages (Fig. 5, lanes d, f, and h). Treatment with IFN-γ on A24 mouse macrophages co-cultivated with CHO cells expressing vector alone increased NO production by about three-fold (Fig. 5, compare lanes i and j). Interestingly, the expression of MV glycoproteins on CHO cells influenced the levels of IFN-γ–induced NO production from A24 macrophages. In particular, co-expression of both the H and F proteins of MV on CHO cells increased NO production from A24 macrophages upon IFN-γ induction (Fig. 5, lane p), whereas the expression of either H or F protein independently did not significantly increase IFN-γ–induced NO production (Fig. 5, lanes l, n, and j, respectively). Furthermore, deletion of the CD46 cytoplasmic domain (Fig. 5, lanes r, t, v, and x) or replacing CYT1 with CYT2 (data not shown) reduced the levels of NO induced by IFN-γ and completely abrogated the enhancing effects on NO production on co-cultivation with CHO cells expressing MV glycoproteins. Taken together, these findings suggest that the reported enhancement of NO production by human CD46 (*21, 22*) is attributable to the CYT1 isoform–mediated cellular response in human macrophages, and purified crosslinking material is sufficient for the IFN-γ–mediated augmentation of NO production.

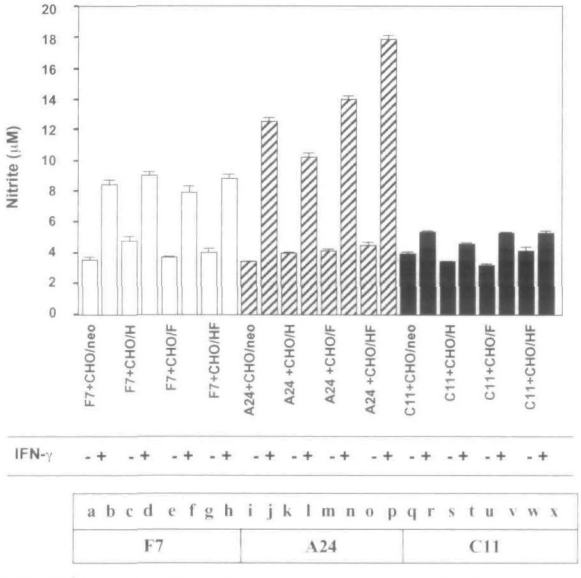


Fig. 5. **NO production from mouse macrophages expressing CD46 co-cultivated with CHO cells expressing MV glycoproteins.** Equal numbers (2×10^6) of mouse macrophages F7, A24, or C11 and CHO cells expressing MV proteins H (H24), F(F21), H plus F (HF15), or neo^R (control) were incubated in the presence (+) or absence (–) of 100 U per ml of IFN-γ. Twenty-four hours after co-cultivation, the culture medium was collected and assayed in triplicate for nitrite accumulation. Solid bars atop each column represent standard deviations of three independent measurements.

DISCUSSION

This study demonstrates that crosslinking of the CYT1 isoform on mouse macrophages with CD46-specific antibody, its $F(ab')_2$ fragment, C3b multimer or MV H and F enhances IFN-γ–induced NO production. This output is independent of viral replication, although the augmentation of NO production appears to be more efficient if infection is accompanied by CD46 stimulation (*21*). Notably, this response was barely observed in CYT2 isoform–expressing cells. These results provide strong evidence that CYT1 and CYT2 of CD46 act differently to regulate cellular responses to modulate the activation of innate immune-competent cells, macrophages and dendritic cells, and that the purified reagents, but not infection, can induce CD46-mediated immune responses. Human macrophages should produce NO in response to CD46 ligands, since they express both CYT1 and CYT2 of CD46 (see Table I).

Three lines of evidence indicate that CD46-specific antibody enhances the NO response by interaction with CD46 independently of Fc receptor signaling. First, control antibody did not enhance IFN-γ–induced NO production in these mouse macrophages. Second, treatment with CD46-specific $F(ab')_2$ plus $F(ab')_2$ of secondary antibody similarly enhanced NO production in mouse macrophages expressing human CYT1 CD46, but not CD46-negative mouse macrophages (Fig. 2). Finally, crosslinking a human CD46 mutant that lacks cytoplasmic domains on mouse macrophages with CD46-specific antibody or $F(ab')_2$ fragment failed to enhance IFN-γ–induced NO production (Figs. 1 and 2). In the same system, CYT2 CD46 slightly inhibited IFN-γ–induced NO production. These results strongly suggest that the ligation of CYT1 CD46 produces a signal that augments IFN-γ–dependent NO production and that the cytoplasmic domains of CD46 are critical for this function.

The point of this study is that the modulation of IFN-γ–induced NO production by CYT1 of CD46 was reproduced with its natural ligands, C3b multimers. Furthermore, a similar tendency was observed with MV glycoproteins expressed on CHO cells. Only the ligation of CD46, irrespective of ligand species, is needed to induce NO production. As RAW cells express mouse CR1 and CR3 in addition to CRRY (data not shown), C3b may elicit cellular responses through these receptors. Mouse CR1 and CR3, however, barely bind to human C3b or its multimers (*36*). Thus, under our conditions, human C3b is likely to interact preferentially with human CD46 expressed on RAW cells. MV H was shown to bind selectively SCR1 and SCR2 domains of CD46 (*32, 34*), and the other SCRs and the ST portions with *O*-linked sugars were shown to modulate MV binding (*4, 37*). On the other hand, C3b binds SCR2, SCR3, and SCR4 (*34, 38*). These results, together with the finding that both M160 (recognizing SCR3) and M177 (recognizing SCR3) (*34*) exert enhancing effects on NO production (*24*), suggest that the clustering of CD46 rather than MV-mediated fusion or crosslinking of specific portions or residues in

CD46 is important for modulating IFN-γ–induced NO production in macrophages. Additional reported features of macrophages in association with MV, binding such as morphological alteration (*24*), augmented adhesiveness (*39, 40*) and down-regulation of CD46 (*41*), may be related to the signaling specific to either CYT1 or CYT2.

The expression of human CD46 in mouse macrophages may have revealed a potentially novel function of CD46 in host defense. The natural ligands for CD46 are C3b and C4b in the alternative and classical complement activation cascades, respectively (*1–3*). We hypothesize that CD46 may play a role on macrophages of enhancing anti-microbial responses against complement-opsonized microbes. This idea can be reinforced by the observations that the C3b multimer mimics C3b-coated microbes (*42, 43*), and that the C3b multimer but not monomer induces efficient CYT1-mediated macrophage activation when the levels of surface-expressed CD46 are low (Fig. 3). Again, clustering but not simple binding should be essential for CD46-mediated macrophage activation. Notably, both the monomer and multimer bind CD46 and are cleaved by factor I into C3bi resulting in the generation of CR3/4 binding sites (*43*). In this regard, CD46 may be a ligand supplier for CR3/4 and in concert with CR3/4 may facilitate the activation of macrophages to a state sufficient for killing internalized viruses in response to IFNs (*44, 45*).

CD46 is a receptor for laboratory-adapted MV (*19, 20*). The expression of human CD46 in most rodent cells facilitates infection by MV strains that utilize CD46 as a receptor (*19, 20, 45, 46*). However, the expression of human CD46 in mouse macrophages does not render these cells permissive to MV infection (*47*). In contrast, the expression of human CD46 restricts MV replication in mouse macrophages (*21, 22*). This restriction of MV replication in mouse macrophages can be dramatically reduced by deleting the cytoplasmic domain from human CD46 (*21, 23*) or replacing CYT1 with CYT2, which slightly attenuates NO production in response to IFN-γ (Fig. 1). Thus, the interaction with CD46 may be detrimental to MV survival in macrophages. In fact, our previous results using human dendritic cells (*35*) and macrophages (*24*) support this assumption. It is noteworthy that only attenuated MV strains, including the measles vaccines, recognize CD46 as a receptor, whereas wild-type MV utilizes CDw150 (SLAM) or as yet unidentified receptors (*48*). In line with this, only CD46-dependent MV strains fail to be efficiently replicated in macrophages, presumably due to the induction of NO (*21, 24, 35*).

The H protein of MV, which binds CD46 to allow virus entry into human cells, exists as a tetramer (*49*). Theoretically, the H tetramer can interact with multiple CD46 molecules. However, we have found that mouse macrophages expressing human CD46 do not produce higher levels of NO upon contact with CHO cells expressing the MV H protein alone (Fig. 5). Apparently, co-expression of both the H and F proteins of MV is necessary to enhance IFN-γ–induced NO production (Fig. 5). This point was confirmed using human macrophages and H/F-expressing cells (*24*). The MV glycoproteins expressed from cloned genes enhance macrophage NO production much less efficiently than infection by MV (Fig. 5, compared to Ref. *21*). It is possible that MV-infected cells synthesize higher levels of viral glycoproteins than transfected CHO cells. In CHO cells, the H and F proteins of MV may accumulate inside the cells to lower NO production by forming complexes with proteins related to NO production, or less efficiently form a functional complex important for interaction with other cell-surface molecules (*50*). On the other hand, the MV glycoproteins may be clustered together during virion assembly on MV-infected cells. Thus, the crosslinking CD46 molecules on MV-infected cells is likely to induce NO more efficiently than when the viral glycoproteins are expressed from cloned genes on uninfected cells.

MV also produces other viral products, such as double-stranded (ds) RNA, known to enhance IFN-γ–induced NO production in mouse macrophages (*21, 22*). Recent studies by us and other groups revealed that Toll-like receptor 3 (TLR3) serves as a signaling receptor for dsRNA (*29, 51, 52*), which differentiates immature dendritic cells to mature stage cells for efficient antigen presentation (Matsumoto and Seya, unpublished data). The combined stimulation of TLR3 and CD46 may occur in measles infection, which may participate in the marked immune suppression associated with measles. We call this the "two receptor hypothesis," confirmation of which requires further studies.

CD46 enhances NO production mainly by augmenting the effects of IFN-γ. IFN-γ acts synergistically with LPS or dsRNA to enhance NO production in mouse macrophages, but LPS does not act synergistically with dsRNA, suggesting that LPS and dsRNA induce NO by partly overlapping pathways that are distinct from that activated by IFN-γ (*53, 54*). However, this response is not dependent on the expression of human CD46 (*21*). The mouse iNOS gene promoter contains NF-κB binding sites responsive to LPS, as well as IFN-stimulable response elements (ISRE) responsive to IFN-γ (*54*). dsRNA induces NO through the activation of PKR and the transcription factor NF-κB (*55*). More recently, we showed that type 1 IFN can be induced *via* TLR3 and an as yet unknown pathway introduced by an unidentified adaptor molecule (*52*). MV has been reported to activate NF-κB in human B cells and glioblastoma cells (*16, 56*). This raises the possibility that MV may induce iNOS expression by pathways that lead to NF-κB activation. The mechanisms by which CYT1 CD46 augments NO response remain to be determined. CYT1 possesses potential phosphorylation sites for protein kinases C and casein kinase 2 (*57, 58*), while CYT2 possesses potential phosphorylation sites for Src kinases and casein kinase 2 (*57*). Subsequent studies suggested that CYT1 recruits Dlg4 (*59*) and probably SHP-1 (Kurita-Taniguchi, Seya *et al.*, unpublished), while CYT2 activates Src family proteins (*60*). Further studies are needed to determine what role, if any, interactions with macrophage kinases play in CD46 tail-mediated macrophage activation.

Humans and monkeys ubiquitously express CD46 of CYT1 and CYT2 homologues (*61*). In contrast, rodents express a single form of CD46 with a short cytoplasmic tail exclusively in the testis (*61*). In parallel, MV infects only primates and induces immune suppression. Our current study indicates that crosslinking human CYT1 CD46 expressed on mouse macrophages enhances IFN-γ–inducible NO production in response to C3b multimer or H/F proteins of MV. Thus, isoform-specific regulation of immune responses is accomplished by the purified ligand material and CD46 in primate innate immune cells. Similar functional differentiation of CD46 isoforms may actually func-

tion in T (*12*) and B lymphocytes (*11*) as well as in the innate immune system, because in primates, multiple isoforms of CD46 are widely expressed including on lymphocytes and innate immune cells.

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