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Molecular Characterization of Human 4Ig-B7-H3, a Member of the B7 Family with Four Ig-Like Domains¹ **FREE**

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Molecular Characterization of Human 4Ig-B7-H3, a Member of the B7 Family with Four Ig-Like Domains¹

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In an effort to characterize molecules with immunoregulatory potential, we raised mAbs to human dendritic cells. We selected an Ab that recognizes a molecule that is induced on monocytes differentiated in vitro toward dendritic cells. Retroviral expression cloning identified this molecule as B7-H3, a member of the B7 family described recently. In contrast to an earlier report, in which B7-H3 was described as a molecule consisting of two Ig-like domains, our cDNA encoded a type I membrane protein with four Ig-like domains, and the molecule identified by us was therefore named 4Ig-B7-H3. mRNA analysis as well as Western blotting experiments performed by us did not reveal evidence for a small B7-H3. B7-H3 is not expressed on peripheral blood lymphocytes, monocytes, or granulocytes. Upon in vitro stimulation, the expression of B7-H3 is induced on T cells, B cells, and NK cells. A number of different approaches were used to investigate the function of human B7-H3. In contrast to an earlier report, our data do not support a costimulatory role of B7-H3 in anti-CD3-mediated activation of the TCR-complex resulting in T cell proliferation and IFN- γ production. *The Journal of Immunology*, 2004, 172: 2352–2359.

In vitro differentiation of monocytes toward dendritic cells (DC)³ results in a dramatic increase in their immune stimulatory capacity (1, 2). In addition, DC can exert potent immunoregulatory effects on T cells (3–5). Therefore, proteins that are induced upon DC differentiation can be considered candidates for molecules with costimulatory or inhibitory properties. By immunizing mice with human monocyte-derived DC (mdDC), we have generated a number of mAb reacting with DC surface structures. One of our Abs, 7-517, binds strongly to mdDC, but does not react with monocytes, granulocytes, or peripheral lymphocytes. Retroviral expression cloning was used to isolate a cDNA encoding the 7-517 Ag from a library constructed from mdDC. Sequence analysis identified it as B7-H3, a B7-like molecule recently identified upon database searches for sequences with homologies to B7 molecules (6). Like most other members of this family, B7-H3 was described as a type I membrane protein with two Ig-like domains. A fusion protein was used for functional characterization and to demonstrate the expression of a B7-H3 counter-receptor on activated, but not resting, T cells. B7-H3 was described to costimulate the growth of T cells, enhance the CTL response, and selectively induce IFN- γ production in T cells (6). Murine B7-H3 was also described as a two-Ig molecule, and using a B7-H3-Ig fusion pro-

tein, evidence for a counter-receptor that is induced on T cells upon activation was found (7). Analyzing B7-H3-deficient mice, one group reported enhanced Th1-type responses in vivo, whereas another group found enhanced antitumor immunity in mice challenged with EL-4 cells transfected to express B7-H3 (8, 9).

As we found that the molecule cloned by us encodes a 110-kDa protein with four-Ig-like domains, we termed it 4Ig-B7-H3. We did not find evidence for the two-Ig-B7-H3 molecule described previously (6). Blocking of B7-H3 on DC with mAbs did not affect the T cell stimulatory capacity of these cells. Furthermore, our results did not confirm that B7-H3 together with anti-CD3-mediated activation of the TCR complex is able to induce T cell proliferation and production of IFN- γ .

Materials and Methods

Abs and cell culture

The mAbs 7-517, 6-311, and 13-I-241 were obtained from BALB/c mice immunized with human mdDC using standard hybridoma technology. The following murine mAbs were also generated in our laboratory: VIAP (isotype control mAb), VIM12 (CD11b), VIM13 (CD14), 4D3 (CD33), and 1/47 (MHC class II). CD3 mAb (UCHT-1) and CD14 mAb (MEM-18) were provided by An der Grub (Kaumberg, Austria). mAb-specific CD19 (BU12) was obtained from Ancell (Bayport, MN), and Abs recognizing CD56 (MEM-188) and CD16 (3G8) were obtained from Caltag Laboratories (Burlingame, CA). mAbs specific for CD64 (32.2) and CD28 (L293) were obtained from American Type Culture Collection (Manassas, VA) and BD Bioscience (Palo Alto, CA), respectively. For stimulation of T cells, the CD3 mAb OKT3 (Ortho Pharmaceutical, Raritan, NJ) was used.

The murine thymoma cell line Bw5147 (referred to as Bw cells throughout this work) and the monocytic cell line Mono Mac 6 (10) were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 10% FBS (Sigma-Aldrich, St. Louis, MO). The ecotropic retroviral packaging cell line Phoenix-E (11, 12), a gift from G. P. Nolan and colleagues (Stanford University, Stanford, CA), and the mouse cell line NIH-3T3 were maintained in DMEM containing 2 mM glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% FBS. PBMC were isolated from heparinized whole blood of healthy volunteer donors by standard density centrifugation with Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). Subsequently, monocytes were isolated by MACS using CD14 mAb VIM13 and MEM-18. Purified T cells were obtained through

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³ Abbreviations used in this paper: DC, dendritic cell; mdDC, monocyte-derived DC; scFv, single-chain fragment of variable region.

depletion of CD11b, CD14, CD16, CD19, CD33, and MHC class II-bearing cells with the respective mAbs. The mdDC were generated by culturing monocytes in RPMI 1640 containing 10% FBS in the presence of human rGM-CSF (50 ng/ml) and human rIL-4 (100 U/ml) for 7–8 days (2). Both reagents were gifts from the Novartis Research Institute (Vienna, Austria). In some experiments DC maturation was induced by cultivating day 5 mdDC for another 2 days in the presence of either LPS from *Escherichia coli* serotype 0127-B8 (1 μ g/ml) or ionomycin (1 μ M; Sigma-Aldrich). For activation of lymphocytes, PBMC were cultivated in the presence of PMA (100 nM; Sigma-Aldrich) and ionomycin (100 nM) for 48 h.

Flow cytometry

For flow cytometric analysis, cells (1×10^7 /ml) were incubated with fluorochrome-conjugated mAb or unlabeled primary Ab (10 μ g/ml) for 20 min on ice and washed. For indirect staining, Oregon Green-conjugated anti-mouse Ig (Molecular Probes, Eugene, OR) was used as a secondary reagent. Staining of FcR-bearing cells was performed in the presence of human IgG Abs (20 mg/ml; Beriglobin, Aventis Behring, Vienna, Austria). For analysis of peripheral DC, PBL were freshly isolated or cultured in the presence of GM-CSF for 48 h and were incubated with biotinylated mAbs to the lineage-specific markers CD3, CD14, CD16, CD19, and a FITC-anti-HLA-DR conjugate. Binding of biotinylated mAbs was detected using a streptavidin-allophycocyanin conjugate (Caltag Laboratories). The expression of the 7-517 Ag was analyzed using PE-labeled mAb 7-517.

Retroviral cDNA expression library

A retroviral cDNA expression library was constructed using mRNA derived from immature DC and mature DC as a source (1.2×10^8 each). Fifty micrograms of retroviral vector pBMN (13) containing the cDNA library was introduced into the ecotropic packaging cells (Phoenix-E) by transfection using Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA). On days 1, 2, and 3 post-transfection, supernatant was harvested. Fifty milliliters of the filtered (0.45- μ m pore size filter) supernatant was supplemented with 50 μ l of polybrene (4 mg/ml), added to 1×10^8 target cells in 60 ml of RPMI 1640 containing 10% FBS. Two days after the last infection cycle the cells were used for the selection procedure.

Isolation of mAb 7-517 Ag-expressing cells from the retrovirus based-cDNA library transduced cell pool

The retrovirus-infected cell pool (1×10^8 cells) was used for the selection procedure with MACS (Miltenyi Biotech, Bergisch Gladbach, Germany) and mAb 7-517 (10 μ g/ml) as previously described (14). Three rounds of magnetic cell sorting were performed. From the cell pool obtained, single-cell clones were established by limiting dilution culturing.

Molecular cloning of the B7-H3 cDNA insert

Genomic DNA was prepared from a 7-517-reactive, single-cell clone using Tri-Reagent (Sigma-Aldrich) following the manufacturer's instructions. The retrovirus-encoded cDNA inserts were PCR-amplified from genomic DNA with the oligonucleotide primers Ban1b (5'-GACCATCTCTA GACTGCCGGATC-3') and Ban2b (5'-CATCCCCCTTTTCTGGA GACTAAATAAAATC-3'), specific for the flanking retroviral sequences. The Expand PCR system (Roche, Mannheim, Germany) was used for the PCR amplification under standard conditions. The obtained PCR products were gel-purified and cloned using Topocloning (Invitrogen). Selected plasmids were transfected into NIH-3T3 cells using Lipofectamine according to the manufacturer's instructions to confirm that the transfected cells react with the mAb 7-517. Plasmid DNA was prepared from selected clones and used for sequence analysis (VBC Genomics, Vienna, Austria).

RT-PCR analysis of B7-H3-mRNA

Total RNA was prepared from different tissues using Tri-Reagent and was used for cDNA synthesis (Superscript First-Strand Synthesis Kit; Invitrogen) according to the manufacturer's protocols. cDNA was amplified using the B7-H3 primers 7517F0 (5'-CACAGGAAGATGCTGCTCG-3') and 7517B3 (5'-CAATGAGACAGACAGACAGC-3'), priming at the beginning of the translated sequence and in the transmembrane region, respectively (250 nM each). cDNA (0.5 μ l for a 20- μ l reaction) was amplified using Reddy PCR Mix (Abgene, Epsom, U.K.) in the presence of 5% DMSO.

Western blotting

Western blotting was performed under standard conditions using B7-H3 mAb 13-I-241 (1 μ g/ml). Bound Ab was detected using HRP-conjugated goat Abs to mouse Ig (DAKO, Glostrup, Denmark; 1/10,000) and chemiluminescence (SuperSignal; Pierce, Rockford, IL).

Allo-MLR using mdDC or Mono Mac 6 cells

For a primary MLR, allogenic, purified T cells (1×10^5 /well) were incubated in a 96-well culture plate with various numbers of immature DC and mAbs (final concentration, 10 μ g/ml). On day 5 [*methyl*- 3 H]thymidine (ICN Pharmaceuticals, Irvine CA) was added to the cultures, and cells were harvested 18 h later. Incorporated [3 H]thymidine was detected on a microplate scintillation counter (Packard Instruments, Meriden, CT). The B7-H3⁻ human monocytic cell line Mono Mac 6 was retrovirally transduced using the 4Ig-B7-H3 cDNA, and 4Ig-B7-H3⁺ cells were isolated by MACS. 4Ig-B7-H3-transduced Mono Mac 6 (MM6 B7-H3) and mock-transduced cells were irradiated (6000 rad) and used to stimulate allogenic MNC. Cocultivation and proliferation measurement were performed as described above. All proliferation assays were performed in triplicate.

Generation of the BwCD64, BwCD64/CD80, BwCD64/4Ig-B7-H3, and BwCD64/2Ig-B7H3 cell lines

Bw cells were retrovirally transduced with the human high affinity FcR (CD64) cDNA, and CD64⁺ cells were selected by MACS. Single-cell clones were obtained from the selected cells. A single-cell clone that showed homogenous expression of CD64 was either mock-transduced (BwCD64 cells) or retrovirally transduced with the human cDNAs encoding 4Ig-B7-H3 or CD80. An expression construct encoding a 2Ig-B7-H3 was also generated by PCR amplifying cDNA encoding the 4Ig-cDNA using short extension times. The smaller PCR product was cloned into the retroviral expression vector pBMN. The obtained product was sequenced to confirm that it encoded a 2Ig-B7-H3 with a IgV₁-IgC₂ domain composition almost identical with that of the B7-H3 sequence reported previously (6), which also encodes an IgV₁-IgC₂ molecule. This construct was also used to transduce BwCD64 cells. From the transduced cell pools, B7-H3⁺ and CD80⁺ cells, respectively, were selected by MACS to obtain the BwCD64/4Ig-B7-H3, BwCD64/2Ig-B7-H3, and BwCD64/CD80 cell lines.

Stimulation of T cells using Ab-loaded BwCD64 cells

Bw cells; BwCD64 cells expressing 4Ig-B7-H3, 2IgB7-H3, or CD80; and control BwCD64 cells were irradiated (6000 rad) and incubated with purified anti-CD3 mAb (final concentration, 10–250 ng/ml) or with mAb to CD3 and CD28 (final concentration, 10 ng/ml each) and added to flat-bottom, 96-well plates (2.5×10^4 cell/well). Purified human T cells (5×10^4) were added to each well. Culture supernatant was harvested after 48 h, and IFN- γ was measured as previously described (15). T cell proliferation was measured after 72 h of coculture with [3 H]thymidine present during the last 18 h. The proliferation assays were performed in triplicate.

Immunosomes

The generation of immunosomes is described in detail.⁴ Briefly, the ectodomains of CD80 and 4Ig-B7-H3 were amplified from a Raji library or the B7-H3 cDNA, respectively, without their leader sequences and were inserted downstream of a CD5 leader and upstream of the CD16 GPI anchor-acceptor into the mammalian expression vector pEAK12 (Edge Biosystems, Gaithersburg, MD). Moloney murine leukemia virus *gag-pol* sequences were cloned into the mammalian expression vector pEAK12. A 293 cell line stably expressing a single-chain fragment of the variable regions (scFv) of the CD3e-specific hybridoma OKT3 attached to the CD14 molecule (OKT3scFv-GPI) was transiently transfected with the expression plasmids described above using the modified calcium-phosphate method (16). Immunosome formation was induced by cotransfecting the *gag-pol* expression vector. Immunosome-containing supernatants were harvested from the transfected 293 cells after 3 days. Cellular debris was removed by centrifugation and filtration through 0.45- μ m pore size syringe filters (Millipore, Billerica, MA) and used directly for the T cell stimulation assays at a dilution of 1/2. The proliferation assays were performed in triplicate.

Results

Reactivity of mAb 7-517

Testing a number of mAbs that were reacting with surface molecules on human mdDC, we identified mAb 7-517 that recognizes a molecule that is not detected on freshly isolated monocytes, but is strongly expressed on immature as well as mature mdDC (Fig. 1). The 7-517 Ag is also induced upon in vitro stimulation of

⁴ S. V. Derdak, E. Bielek, O. Majdic, W. Knapp, B. Seed, and W. F. Pickl. Immunosomes: reconstitution of the antigen-presenting unit on plasma membrane-derived vesicles with high T cell stimulatory activity. *Submitted for publication.*

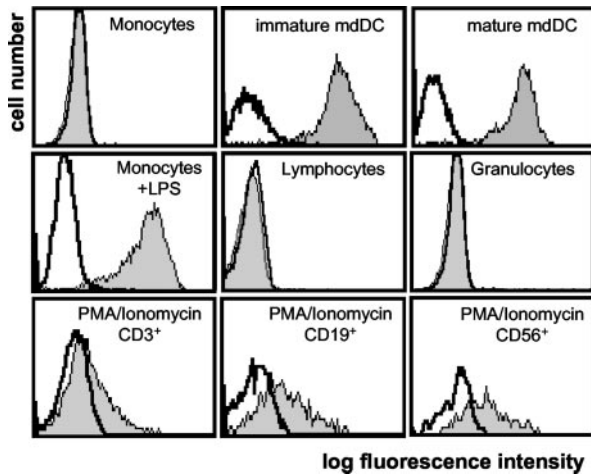


FIGURE 1. Reactivity of mAb 7-517 with freshly isolated and in vitro-stimulated lymphocytes. In vitro-stimulated PBL were double-stained with 7-517-FITC mAb and PE-conjugated CD3, CD19, or CD56 mAbs. Shaded histograms represent reactivity of cells with B7-H3 mAb 7-517; open histograms show reactivity of the isotype control.

monocytes using LPS or the cytokines TNF- α , IFN- γ , and IFN- α in combination with GM-CSF (Fig. 1 and data not shown). mAb 7-517 did not react with peripheral lymphocytes and granulocytes. After 48-h in vitro stimulation of PBL using PMA and ionomycin, the 7-517 Ag was induced in T and B lymphocytes and NK cells (Fig. 1). To evaluate whether mAb 7-517 reacts with peripheral DC, LIN⁻HLA-DR⁺ PBL were analyzed for reactivity with mAb 7-517. This cell population was consistently negative for the mAb 7-517 Ag. After 48-h in vitro culture in the presence of GM-CSF, the Ag recognized by mAb 7-517 was induced in the LIN⁻HLA-DR⁺ cell population (data not shown).

Identification of the 7-517 Ag as B7-H3 by molecular expression cloning

As the staining pattern of mAb 7-517 on primary cells and a number of human cell lines of hemopoietic and nonhemopoietic origin differed from the expression of molecules known to be induced on monocytes differentiated along the DC axis, expression cloning was used to identify the Ag recognized by this Ab. A retroviral cDNA library constructed from human mdDC was expressed in mouse target cells. The transduced cell pool was subjected to three rounds of selection with the mAb 7-517 (Fig. 2A). The three PCR products obtained from a single-cell clone were introduced into an eukaryotic expression vector (Fig. 2, B and C). Cells expressing the protein encoded by the 3.2-kb PCR product were specifically reacting with the 7-517 mAb (Fig. 2D). The 5' end of the cDNA insert, named 7-517-75, was sequenced and was found to be identical with B7-H3, a member of the B7 family cloned recently (6). Two additional mAbs raised in our laboratory (mAbs 6-311 and 13-I-241) were also found to specifically react with cells expressing B7-H3 (data not shown).

DNA sequence analysis

As only the coding cDNA sequence of B7-H3 has been reported to date, the retroviral cDNA insert encoding the mAb 7-517 Ag was subjected to DNA sequence analysis. The insert of 3222 bp contained a short 5'-untranslated stretch and an open reading frame beginning with a start codon encoding 534 aa and a 1528-bp 3'-untranslated sequence followed by a poly(A) tail (Fig. 3A). The nucleotide sequence has been submitted to the EMBL Data Library (accession no. AJ583695). The deduced amino acid sequence con-

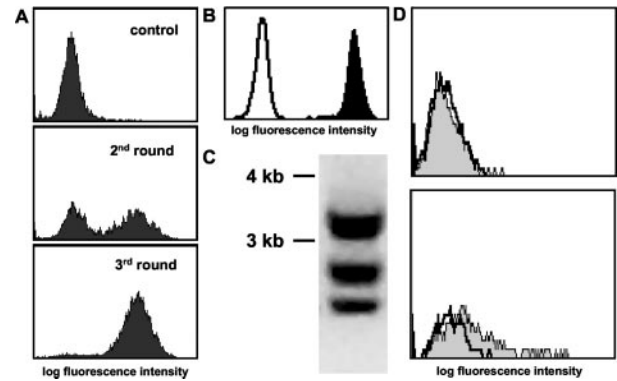


FIGURE 2. Identification of the 7-517 Ag by molecular expression cloning. A, Enrichment of 7-517-reactive target cells. Untransduced target cells (control) and cell pools obtained after two and three rounds of MACS selection of target cells transduced with the retroviral DC expression library were analyzed by flow cytometry. B, A single-cell clone obtained from the cell pool enriched for 7-517-reactive cells was analyzed. C, PCR recovery of the retroviral cDNA inserts from the genomic DNA of the 7-517-reactive cell clone. D, NIH-3T3 cells transfected with plasmid DNA containing the 3.2-kb PCR product (lower panel), but not control-transfected cells (upper panel), react with mAb 7-517 (dark histograms) or isotype control mAb (open histograms) and Oregon Green-conjugated goat anti-mouse Ig Abs as a secondary reagent.

sisted of a short leader sequence and four Ig-like domains, followed by a transmembrane domain and a short cytoplasmic tail (Fig. 3B). The Ig-like domains consisted of two pairs of IgV-IgC domains (Fig. 3B). The sequences of the two IgV-IgC pairs were highly homologous (95% identity; Fig. 3C). In an earlier report the B7-H3 molecule was described as a protein 316 aa in length with two Ig-like domains, an IgV-like and an IgC-like domain (6). We therefore termed the larger B7-H3 molecule with four Ig-like domains cloned by us 4Ig-B7-H3. Established sequence tag data and experimental evidence that pointed to a B7-H3 molecule with four Ig-like domains (B7-H3b) were reported recently (7, 17, 18).

RT-PCR analysis of B7-H3 encoding mRNA derived from different tissues

Next we wanted to investigate whether the short and long forms might be differentially expressed in DC and other cell types. For that, a pair of oligonucleotide primers specific for sequences encoding the leader sequence and the transmembrane region of B7-H3, respectively, was generated and used to PCR-amplify cDNAs from different tissues. The main product in all cDNAs analyzed was a band of 1.3 kb corresponding to the 4Ig-B7-H3 sequence. However, a faint 0.7-kb product, which would correspond to the short two-Ig B7-H3, was also found in some reactions. Interestingly, a minor band of this size was obtained in addition to the expected large band when the cDNA 7-517-75 encoding 4Ig-B7-H3 was used as a template for PCR amplification (Fig. 4). This 0.7-kb band is a PCR artifact resulting from the high homology of the IgV₁-IgC₁ with the IgV₂-IgC₂-sequence in the 7-517-75 cDNA. In addition, PCR products larger than the expected 1.3 kb, probably also resulting from elongation of misprimed, unfinished PCR products, were obtained in some reactions (Fig. 4). The intensity of the bands was dependent on the PCR conditions. When short annealing and extension temperatures were used, only the small band was obtained (data not shown). We therefore suspected that the 0.7-kb products resulting from the RT-PCR amplification of different tissues might also be PCR artifacts. We cloned some of the 0.7-kb products and found that they encoded the 2Ig B7-H3. Due to different cross-over points from the IgV₁-IgC₁ to the IgV₂-

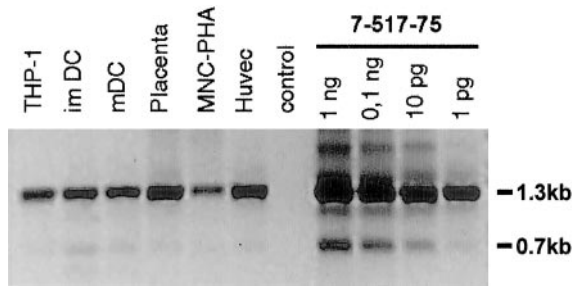


FIGURE 4. RT-PCR analysis of different human RNA samples using B7-H3-specific primers. A product of 1.3 kb corresponds to a 4Ig-B7-H3 molecule with four Ig domains, whereas a 0.7-kb band would represent the 2 Ig B7-H3 protein. The indicated amounts of plasmid DNA containing the 4Ig-B7-H3 cDNA clone 7-517-75 were also used as a template under the same PCR conditions.

transductants (Fig. 5A). An additional 80- to 90-kDa protein detected by mAb 13-I-241 in both Bw transductants is the result of reactivity of mAb 13-I-241 with all Bw cell lysates. However, mAb 13-I-241 does not bind to the cell surface of intact Bw cells that do not express human B7-H3.

In whole cellular lysates derived from mdDC, a band of ~110 kDa was detected by B7-H3-mAb 13-I-241 (Fig. 5B). No smaller band was seen in mdDC, which also points to 4Ig-B7-H3 as the sole or at least prevalent B7-H3 species. Reactivity of mAb 13-I-241 with a putative small form of B7-H3 was confirmed by cloning and expressing a PCR product representing the two-Ig B7-H3 (data not shown).

B7-H3-specific mAb do not affect the T cell stimulatory capacity of human mdDC

B7-H3 is strongly expressed on mature as well as immature mdDC. A counter-receptor for the human as well as the murine B7-H3 was described on activated T cells (6, 7). To evaluate the functional role of B7-H3 on DC, we performed MLR experiments in the presence of B7-H3 mAb. Three mAbs recognizing B7-H3 were raised in our laboratory, and epitope mapping revealed that they recognized different structures on the extracellular domains of B7-H3 (data not shown). As a control we used mAbs specific for CD58 and B7-H1 under the same conditions. Blocking of these structures on APC was previously described to enhance (B7-H1) or reduce (CD58) T cell proliferation (15, 19). As expected, the presence of CD58 mAb or B7-H1 mAb affected the allogenic response of T cells to DC. In contrast, the presence of B7-H3 mAbs

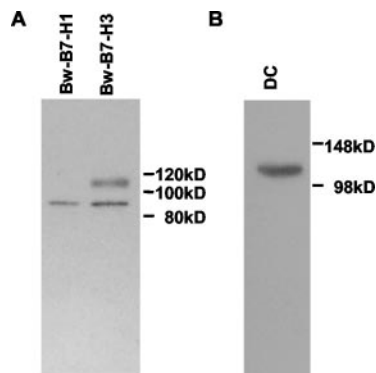


FIGURE 5. B7-H3 mAb 13-I-241 recognizes a protein of ~110 kDa. A, Whole cellular lysates from Bw cells expressing human B7-H1 or 4Ig-B7-H3 were probed with mAb 13-I-241. B, DC whole cellular lysates were used for Western blot analysis with B7-H3 mAb 13-I-241.

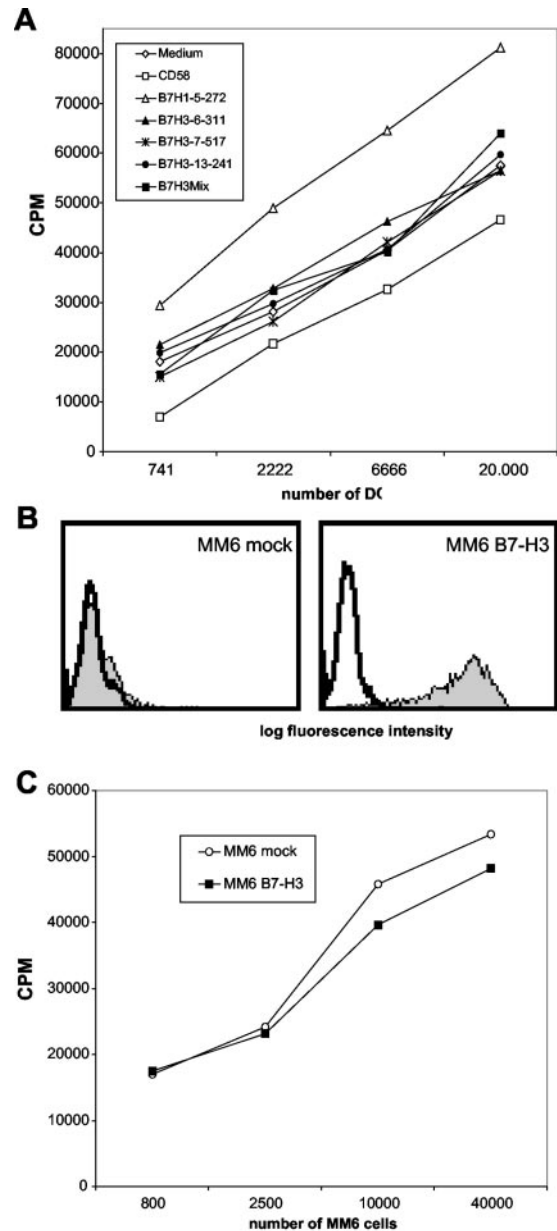


FIGURE 6. The presence of B7-H3 mAb or the expression of 4Ig-B7-H3 does not affect the capacity of APC to stimulate T cells in an Allo-MLR. A, Immature mdDC were incubated with mAb as indicated and then used to stimulate allogenic T cells (1×10^5 /well). Three independent experiments were performed with similar results. B, FACS analysis of mock-transduced (MM6 mock) and 4Ig-B7-H3-transduced Mono Mac 6 cells (MM6 B7-H3) using the B7-H3 mAb 7-517 (gray histograms). The bold outline represents staining using an isotype control mAb. Oregon Green-conjugated goat anti-mouse Ig Ab was used as a secondary reagent. C, B7-H3-transduced and mock-transduced Mono Mac 6 cells were used to stimulate allogenic MNC.

either alone or in combination, had no effect on the proliferation of T cells (Fig. 6A). Day 4 culture supernatants from MLRs were used for the measurement of IFN- γ , IL-4, IL-2, and IL-10. We did not obtain an effect of the B7-H3 mAbs on the release of these cytokines; namely, IFN- γ was not reduced (data not shown). We also performed MLR experiments using mature DC that are strong stimulator cells or a number of B7-H3-expressing myeloid cell lines that evoke a weak allogenic response in T cells. B7-H3 mAbs again had no effect on the capacity of these cells to stimulate allogenic T cells (data not shown).

Expression of B7-H3 in a monocytic cell line does not affect the capacity of these cells to stimulate T cells in an MLR

The monocytic cell line Mono Mac 6 that is B7-H3 negative was retrovirally transduced to express 4Ig-B7-H3 (Fig. 6B). The resulting cells (MM6 B7-H3) and the mock-transduced Mono Mac 6 cells were compared regarding their capacity to stimulate allogenic MNC. We found that the expression of 4Ig-B7-H3 by Mono Mac 6 cells did not affect the stimulatory capacity of these cells (Fig. 6C).

B7-H3 cannot costimulate signal 1 generated by CD3 ligation

Bw cells expressing the human high affinity FcR CD64 (BwCD64) and cells that express both human CD64 and human 4Ig-B7-H3 (BwCD64/4Ig-B7-H3; Fig. 7A) were incubated with anti-CD3 mAb alone or with mAbs to CD3 and CD28 and used to stimulate purified human T cells. In addition, BwCD64/2Ig-B7-H3 and BwCD64/CD80 cells were tested. All three B7-H3-specific mAbs raised in our laboratory showed strong reactivity with the 2Ig-B7-H3 (Fig. 7A and data not shown). BwCD64 cells loaded with

CD3 mAb alone did not elicit a proliferative response in T cells even in the presence of high concentrations of anti-CD3. As expected, CD80 acted costimulatory to anti-CD3 stimulation as BwCD64/CD80 cells induced T cell proliferation in the presence of CD3 mAb. Even in the presence of high amounts of anti-CD3 mAb, BwCD64/4Ig-B7-H3 cells and BwCD64/2Ig-B7-H3 cells were not able to induce proliferation in T cells, indicating that 4Ig-B7-H3 and 2Ig-B7-H3 do not costimulate the triggering of TCR complex mediated by anti-CD3 mAb. In contrast, even very low amounts of CD28 mAb together with CD3 mAb induced strong T cell proliferation in the presence of all CD64-expressing Bw cells. Cross-linking of the mAbs on the surface of Bw cells was required in these experiments, because only Bw cells expressing CD64 were able to stimulate T cells in the presence of CD3 and CD28 mAb, and T cells did not proliferate in the presence of soluble CD3 and CD28 mAbs (Fig. 7B and data not shown). Similar to T cell proliferation, IFN- γ was only detectable in appreciable amounts in cell culture supernatants upon stimulation of T cells with CD3 and CD28 mAbs cross-linked via Bw cells expressing CD64 alone. Whereas CD80 costimulated IFN- γ production of T cells in the presence of CD3 mAb, 2Ig-B7-H3 or 4Ig-B7-H3 did not (Fig. 7C). Compared with BwCD64 cells loaded with anti-CD3 and anti-CD28, the T cell proliferation and IFN- γ production induced by BwCD64/4Ig-B7-H3 and BwCD64/2Ig-B7-H3 in the presence of these mAbs was reduced in some, but not all, experiments performed.

4Ig-B7-H3 on immunosomes is not costimulatory

As an additional experimental system we used immunosomes, i.e., pseudotyped plasma membrane-derived microvesicles, expressing on their surface anti-CD3-scFv alone or in combination with CD80, to investigate the role of B7-H3 for T cell responses. For that purpose human CD80 and 4Ig-B7-H3 were modified with GPI anchor acceptor sequences to be targeted to lipid rafts and were transiently expressed in a human cell line stably expressing an anti-CD3-scFv construct. Formation of immunosomes was induced from the lipid raft area by coexpression of viral capsid proteins.

Immunosomes expressing only anti-CD3-scFv were unable to stimulate human MNC or T cells (Fig. 8A and data not shown). Vesicles coexpressing 4Ig-B7-H3 and anti-CD3-scFv also failed to stimulate PBMC or T cells, indicating again that 4Ig-B7-H3 together with the surrogate TCR ligand are unable to promote T cell growth and IFN- γ production (Fig. 8A and data not shown). High amounts of 4Ig-B7-H3 were expressed in these immunosomes, as confirmed by Western blotting (data not shown). In contrast, control microvesicles coexpressing CD80 together with the anti-CD3-scFv induced strong T cell activation and proliferation (Fig. 8, A and B). Cointroduction of 4Ig-B7-H3 in such microvesicles did inhibit CD80-induced proliferation in most, but not all, experiments (Fig. 8B). The presence of comparable amounts of CD80 in the vesicle fractions was confirmed by Western blotting (data not shown).

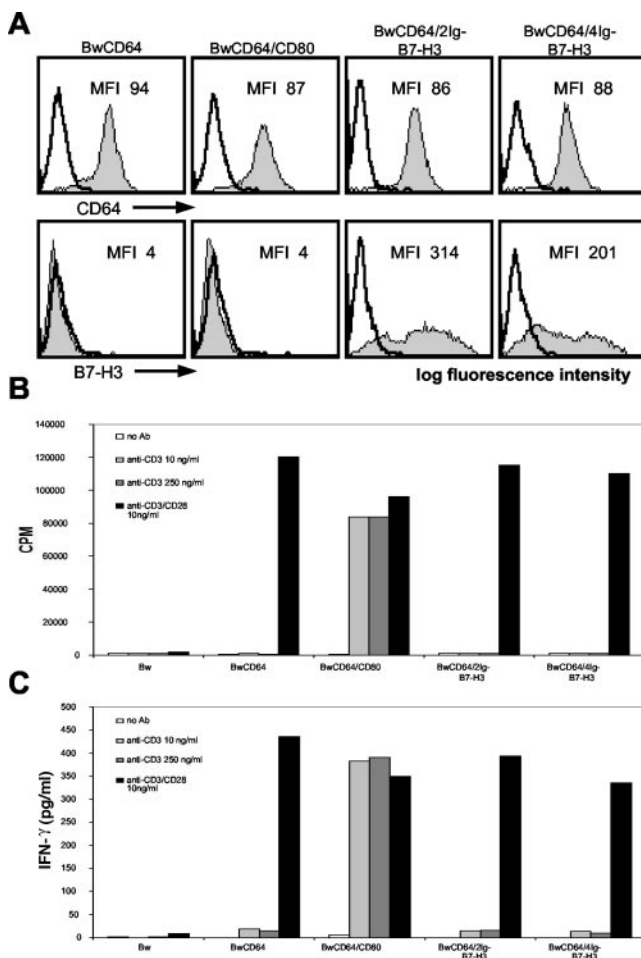


FIGURE 7. B7-H3 cannot costimulate anti-CD3 activation of T cells. *A*, FACS analysis of BwCD64, BwCD64/CD80, BwCD64/2Ig-B7-H3, and BwCD64/4Ig-B7-H3 cells using mAbs specific for CD64 and B7-H3 (gray histograms). Open histograms represent reactivity of the isotype control. *B*, Irradiated BwCD64, BwCD64/CD80, BwCD64/2Ig-B7-H3, and BwCD64/4Ig-B7-H3 cells were loaded with Abs as indicated and cocultured with purified human T cells. For control purposes, T cells were also stimulated with mAb in the presence of the parental Bw cell line (CD64⁻). *C*, Cell culture supernatant derived from the experiment shown in *B* was used for measurement of IFN- γ . Data from one experiment representative of three independent experiments are shown.

Discussion

In the current study we have used retroviral expression cloning and our mAb 7-517 to identify a B7-H3 form with four-Ig-like domains (4Ig-B7-H3). Chapoval and co-workers (6) described B7-H3 as a molecule with two-Ig-like domains. To clarify this discrepancy, we performed RT-PCR analysis as well as Western blotting experiments, which pointed to 4Ig-B7-H3 as the sole or at least prevalent form of B7-H3. All B7-H3 mAbs raised in our laboratory reacted strongly with a 2Ig-B7-H3 molecule that was generated by us for control purposes. This indicates that the results obtained in our Western blotting experiments, which only gave

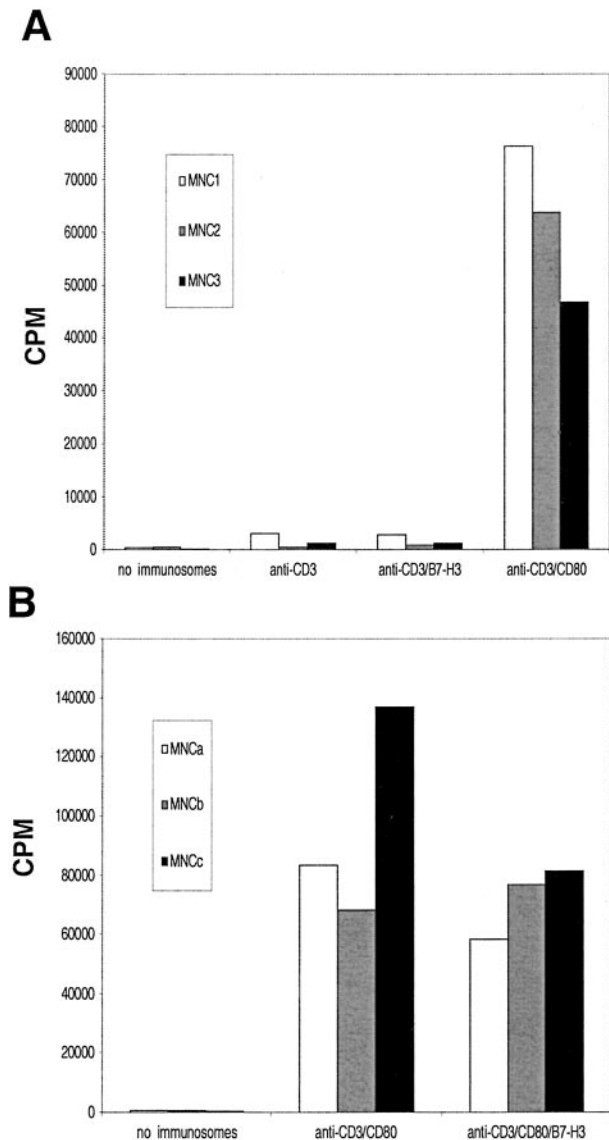


FIGURE 8. Presence of B7-H3 in anti-CD3-scFv-containing vesicles is not costimulatory. *A*, Immunosomes expressing the indicated molecules on their surface were used to stimulate human MNC derived from three individuals. *B*, The effect of 4Ig-B7-H3 coexpression on immunosomes expressing anti-CD3 and -CD80 on MNC of three individuals is shown. The data shown are representative of at least three independent experiments.

evidence for 4Ig-B7-H3, are not due to the inability of our mAb to recognize a putative 2Ig-B7-H3. Furthermore, database searches revealed no EST clones pointing to the short (two-Ig) B7-H3. As Northern blot analysis of a number of different tissues and cell lines clearly detected a single B7-H3 mRNA that corresponds well to the size of the full-length cDNA reported in this study, we suggest that the name B7-H3 be assigned to the four-Ig protein. Recently, RT-PCR-based evidence that the 4Ig-B7-H3 is prevalent over the small form in a number of human tissues was reported (18). In light of the results that were obtained upon PCR amplification of sequences encoding the full-length B7-H3 (Fig. 4), it cannot be excluded that the small products pointing to a 2Ig-B7-H3 obtained in this study were PCR artifacts.

Human B7-H3 is encoded on chromosome 15, and the four-Ig structure of B7-H3 is likely to be the result of a duplication of the locus, which encodes the B7-H3-IgV and -IgC exons. This duplication can be considered a relatively recent event in evolution,

because the two pairs of IgV-IgC sequences are highly homologous. Recently, B7S1, a new B7 homologue, was described that, in being a GPI-linked molecule, also differs from the domain composition of the other known members of this family (20).

We found B7-H3 to be strongly expressed on both immature as well as mature mDC. In MLR experiments the presence of three mAb recognizing three different epitopes on the B7-H3 molecule did not alter the stimulatory capacity of DC. In contrast to the B7-H3 mAbs, the presence of any of the B7-H1-mAbs raised in our laboratory (three mAbs recognizing two different epitopes) strongly enhanced the T cell stimulatory function of DC (Ref. 15 and our unpublished observations). The expression of B7-H3 on a monocytic cell line also did not significantly alter the allogenic T cell response to these cells. Therefore, it seems unlikely that the lack of effect seen with our mAbs is due to their inability to block the function of B7-H3. It is conceivable, however, that blocking of B7-H3 on DC does not have a significant effect due to the redundancy of other costimulatory molecules on these cells. We therefore tried to elucidate a putative costimulatory effect of B7-H3 in alternative experimental systems. To this end we used mouse cells that can be loaded with stimulatory mAbs via the human high affinity FcR CD64. When loaded with anti-CD3 mAbs, the proliferation of human T cells was not induced, indicating that there are no molecules on the surface of these cells that can costimulate CD3-mediated TCR activation (signal 1). Upon expression of B7-H3 on these cells, T cell proliferation still could not be induced in the presence of anti-CD3 mAbs, demonstrating that B7-H3 is not able to costimulate CD3 ligation of purified human T cells. To test whether the previously described 2Ig variant of B7-H3 might differ in its costimulatory activity from the full-length B7-H3, both 4Ig-B7-H3 and a small B7-H3 molecule were tested side by side in our system. (Fig. 7). Similar results were obtained when we used a reductionist version of the plasma membrane, i.e., immunosomes, expressing an anti-CD3-scFv and B7-H3 (Fig. 8). Immunosomes coexpressing the two-Ig-B7-H3 molecule together with anti-CD3-scFv also failed to stimulate T cell proliferation (data not shown). Our findings regarding the lack of costimulatory function of B7-H3 differ from an earlier report in which B7-H3-Ig fusion proteins and low amounts of CD3 mAbs were immobilized on plastic surfaces. In these experiments B7-H3-Ig enhanced T cell growth in a dose-dependent manner (6).

Contradictory results regarding the costimulatory role of B7 homologues were reported previously in the case of the PD-1 ligands PD-L1 and PD-L2 (21–25). As discussed by Brown and colleagues (19), some of these discrepancies might be due to the use of plate-bound Fc fusion proteins, which when present at high density might act as antagonists rather than as agonists. For this reason we had focused on alternative experimental systems to investigate the function of the B7-H3 molecule.

In none of our experiments was evidence for a specific induction of IFN- γ through B7-H3 obtained, whereas Chapoval and co-workers (6) reported that B7-H3 specifically induces IFN- γ production in T cells. In one of their experiments they found that irradiated 293 cells transfected with a B7-H3 expression vector strongly induced IFN- γ production in PBL after 96 h of coculture. It is interesting to note that in this experiment mock-transfected 293 did not induce IFN- γ production, although using our B7-H3 mAbs we found 293 cells to endogenously express high levels of B7-H3 (data not shown).

The B7 homologues reported to date have counter-receptors on T cells, which are either costimulatory (ICOS) or inhibitory (PD-1) (23, 26–28). The receptor of B7-H3 is not yet known, but it is conceivable that it also can affect the stimulation of T cells or other lymphocytes under certain conditions. A possible functional role

of B7-H3 could have important implications, because B7-H3 is widely expressed on nonhemopoietic tissues. Although the present study does not support a costimulatory role for B7-H3 in primary stimulation of T cells, it is possible that B7-H3 does functionally act on T cells in a context that was not investigated in this study. For instance, evidence for an enhanced CTL response to B7-H3-expressing melanoma cells and increased rejection of murine tumor cells expressing B7-H3 was reported previously (6, 9). As a counter-receptor for B7-H3 appears to be induced on T cells upon activation, we also investigated the effect of B7-H3 on preactivated T cells. However, the presence of B7-H3 mAbs on DC did not affect their capacity to stimulate previously activated T cells (our unpublished observations). We found in most, but not all, experiments that the presence of B7-H3 on Bw cells or immunostimulatory vesicles led to a slight reduction of T cell proliferation and cytokine production induced by stimulation of the TCR complex and CD28 (Figs. 7 and 8). Data that pointed to a down-regulation of human T responses by B7-H3 have been reported recently (18). This could indicate that B7-H3, like B7-H1 and PD-L2, binds an inhibitory receptor on T cells. However, further studies are needed to provide evidence for an inhibitory role of B7-H3. Identification of a B7-H3 ligand on T cells would facilitate the functional analysis of this new member of the B7 family of molecules.

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