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A Previously Unrecognized Protein-Protein Interaction between TWEAK and CD163: Potential Biological Implications[1]

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TWEAK (TNF-like weak inducer of apoptosis) is a TNF superfamily member implicated in several mechanisms. Although fibroblast growth factor inducible 14 (Fn14)/TweakR has been reported as its receptor, an as yet unrecognized surface molecule(s) might modulate TWEAK function(s). Thus, we set out to identify TWEAK-binding proteins by screening a combinatorial peptide library. Cyclic peptides containing a consensus motif (WXDDG) bound to TWEAK specifically. These peptides were similar to CD163, a scavenger receptor cysteine-rich domain family member, restricted to the monocyte/macrophage lineage and responsible for the uptake of circulating haptoglobin-hemoglobin (Hp-Hb) complexes. Sequence profile analysis suggested that TWEAK mimicked the CD163 natural ligand (Hp-Hb). Consistently, we show dose-dependent TWEAK binding to CD163 and blockade by an anti-CD163 Ab. In a competition assay, both soluble CD163 and Fn14/TweakR were able to compete off TWEAK binding to coated Fn14/TweakR or CD163, respectively. Flow-cytometry and immunofluorescence assays showed that human monocytes (Fn14/TweakR negative and CD163 positive) bind TWEAK, thus blocking the recognition of CD163 and reducing the activation mediated by a specific mAb in these cells. We demonstrate that monocytes can sequester TWEAK from supernatants, thus preventing tumor cell apoptosis; this effect was reverted by preincubation with the peptide mimicking CD163 or with a mAb anti-CD163, indicating specificity. Finally, we show that recombinant human TWEAK binding to CD163-transfected Chinese hamster ovary cells is inhibited by the presence of either unlabeled TWEAK or the Hp-Hb complex. Together, these data are consistent with the hypothesis that CD163 either acts as a TWEAK scavenger in pathological conditions or serves as an alternate receptor for TWEAK in cells lacking Fn14/TweakR. ***The Journal of Immunology,*** **2007, 178: 8183–8194.**

The TNF and TNFR superfamilies play important roles in the development and regulation of the immune system and are implicated in diverse physiologic processes such as apoptosis, cell proliferation, and bone remodeling (1–3). TWEAK (TNFS12) is a "TNF-like weak inducer" of apoptosis (hence its name) through a non-death domain-dependent mechanism (Refs. 2 and 4; see also HBNC Gene Family Nomenclature; http://www.gene.ucl.ac.uk/nomenclature/genefamily/tnftop.html). It also mediates angiogenesis (5, 6) and inflammation (7, 8). In particular, interactions between TWEAK and monocytes have been reported (9, 10).

However, despite this relatively extensive literature, the complete biological effects of TWEAK remain largely unknown because cells lacking the corresponding receptor (11–13) have also been shown to be TWEAK sensitive. Death receptor 3 (DR3; TNFRS12), a member of the TNFR family containing an intracellular death domain, was initially suggested as a TWEAK-binding molecule (14, 15), but subsequent studies opposed these results (12). More recently, fibroblast growth factor inducible 14 (Fn14)/TweakR has been reported to control TWEAK-associated proliferation of endothelial cells and angiogenesis (16). Supporting these findings, Nakayama et al. have shown that Fn14/TweakR mediates TWEAK-induced programmed cell death (17). However, because Fn14/TweakR lacks the cytoplasmic death domain, the ligand-receptor structural basis is still unclear. Finally, Polek et al. (18) have demonstrated that TWEAK mediates signal transduction and linear differentiation of monocyte/macrophage cells lacking Fn14/TweakR, suggesting that such cells contain an alternative TWEAK receptor, TweakR2. Interestingly, TweakR2 and Fn14/TweakR induce a differential activation of signaling pathways (18). Nevertheless, the identification of such a putative receptor remains elusive.

In this study, we report the screening of a random combinatorial peptide library (19–23) on TWEAK to identify new TWEAK-binding proteins. We show that TWEAK binds specifically to several consensus peptide motifs that are homologous to the scavenger receptor

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cysteine-rich (SRCR)[5] CD163 protein (also called M130 and RM3/1; Refs. 24–28). Consistently, TWEAK binding to purified CD163 was dose dependent and blocked by either the peptide motif mimicking CD163 or by an anti-CD163 mAb. Interestingly, in competition assays TWEAK binding to Fn14/TweakR is competed out by preincubation with soluble CD163 and vice versa. Next, we used cells sensitive to TWEAK but Fn14/TweakR deficient (CD14-positive human monocytes) to demonstrate that TWEAK interacts with CD163 expressed on the cell surface. This association can partially inhibit the activation of monocytes by an anti-CD163 Ab. Then, we show that TWEAK-mediated apoptosis in IFN-γ-treated HT29 cells (12) can be prevented when TWEAK is preincubated with monocytes but not when an anti-CD163 mAb or the peptide that mimics CD163 is added to the culture medium. Finally, we demonstrate that CD163-transfected Chinese hamster ovary (CHO) cells bind ^{125}I-labeled recombinant human TWEAK (^{125}I-rHuTWEAK), but other SRCR members (CD5 and CD6) do not. This process is reverted in a dose-dependent manner by the addition of unlabeled TWEAK; haptoglobin (Hp) and hemoglobin (Hb) complex (Hp-Hb); peptide mimicking CD163 and anti-CD163 Ab. Together, these findings indicate a possible functional link between TWEAK and CD163.

Materials and Methods

Reagents

rHuTWEAK and the goat polyclonal anti-human TWEAK Ab were purchased from Peprotech. CD163 was purified from solubilized human spleen membranes as described (29). Anti-CD163 Abs were obtained as follows: clone GHI/61 from BD Biosciences, clone Ki-M8 from BMA Biomedicals, clone10D6 from Vector Laboratories, and clone AM-3K as described (30). Secondary Abs used were HRP-conjugated anti-goat IgG from Sigma-Aldrich and HRP-conjugated anti-mouse IgG from Bio-Rad. FITC-conjugated anti-human CD14 was purchased from Miltenyi Biotec, purified and PE-conjugated anti-Fn14/TweakR (PE-ITEM-4) and anti-human CD5 (clone UCHT2) Abs were purchased from eBiosciences, and Cyanine 3 (Cy3)-conjugated AffiniPure F(ab′)$_2$ donkey anti-goat IgG (H+L) was purchased from Jackson ImmunoResearch Laboratories. Isotype controls (PE-mIgG1/k, PE-mIgG2b/k, and FITC-mIgG2a) and the anti-human CD6 Ab (clone M-T605) were purchased from BD Pharmingen. CWDDGWSFC (CD163-like peptide) and CRKFRDEATC (used as a control peptide) were purchased from Genemed Synthesis. Human epidermal growth factor (EGF), human IL-2, and human IL-11 and their correspondent Abs (goat anti-human) were from R&D Systems. Human Hb and Hp phenotype 2-2 were from Sigma-Aldrich. FuGENE (Roche) was used for transfection procedures. Na^{125}I (3.7 GBq/ml) was purchased from GE Healthcare Bioscience. Human CD163 (transcript variant 1)-pCMV6-XL4, Fn14-pCMV6-XL5, and human CD5 full-length cDNA clones were purchased from OriGene Technologies, and human CD6 was from Invitrogen Life Technologies.

Phage display screening and phage binding assays

A phage display random peptide library based on the vector fUSE5 displaying the insert CX$_7$C (where C is cysteine and X is any residue) (23) was used in the screening (31, 32); phage input was 2.5×10^9 transforming units (TU). The TWEAK-GST recombinant fusion protein (18) was coated on microtiter wells (Linbro; ICN Pharmaceuticals) as described (21) at 4.5 μg per 50 μl of PBS. The original cyclic phage peptide inserts of seven amino acid residues were analyzed by multiple sequence alignment by using the Clustal W software (European Molecular Biology Laboratory; 32, 33). The selected motifs were then searched against protein databases (National Center for Biotechnology Information (NCBI); www.ncbi.nlm.nih.gov/BLAST/) to search for similarity to known human proteins. Phage binding assays on rHuTWEAK, TWEAK-GST, GST, and BSA (Fraction V, Sigma-Aldrich) were conducted as described (21). Briefly, proteins were immobilized on microtiter wells overnight at 4°C at decreasing concentrations (from 5.0 to 0.5 μg/well). Wells were washed twice with PBS, blocked with PBS plus 3% BSA for 2 h at room temperature, and incubated with 10^9 TU of CWDDGWSFC, CLWTDDGYC, CYWGDDGFC, CWWFDDGYC, and CRWADDGFC phage clones or insertless fd-tet phage in 50 μl PBS containing 2% BSA. After 1 h at room temperature, the wells were washed 10 times with PBS and bound phage was recovered by bacterial infection. Goat polyclonal anti-human TWEAK Ab (Peprotech) at 2 μg/ml was used to evaluate the inhibition of phage binding for 90 min at room temperature. In an alternative experimental design, either CWDDGWSFC phage or fd-tet was coincubated with mouse anti-human CD163 mAb (clone GHI/61; 10 μg/ml) or a mouse IgG1 control isotype and then added to rHuTWEAK or BSA-coated wells.

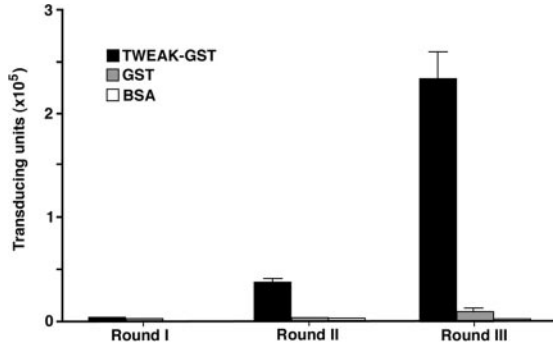

FIGURE 1. In vitro screening for peptides targeting TWEAK from a random phage display library. Microtiter plates coated with TWEAK-GST fusion protein were incubated with 2.5×10^9 TU of a CX$_7$C phage display random peptide library. Bound phage was recovered and amplified for the subsequent second and third rounds of selection on TWEAK-GST, GST, and BSA. Bound phage was quantified by counting TU plated in serial dilutions. Bars, mean ± SEM from duplicate wells.

Protein-protein interaction assays

CD163 or Fn14/TweakR was coated onto a 96-well plate at 2 μg/ml and rHuTWEAK was added at 2 μg/ml. VEGF and BSA were used as coating negative controls. After 2 h, the binding of TWEAK to CD163 or Fn14/TweakR was detected by ELISA with an anti-TWEAK Ab (diluted at 1/1,000). Parallel ELISAs were performed by using a 1/150 dilution of an anti-CD163 Ab followed by an HRP-conjugated anti-mouse IgG to confirm CD163 coating or by adding EGF, IL-2 or IL-11 as alternate ligands for CD163 and recognizing the cytokines with the corresponding Abs. Bound proteins were analyzed by ELISA on an automated reader at 450 nm (Bio-Tek Instruments). For experiments showing dose-dependent TWEAK binding to CD163 or Fn14/TweakR, increasing concentrations of rHu TWEAK were added to the wells (3, 10, 30, 100, 300 and 1,000 ng/ml). In competition experiments, soluble CD163 or soluble Fn14/TweakR (at a range from 0.05 μM to 15 μM) was preincubated with rHuTWEAK for 30 min and then added to the coated Fn14/TweakR or CD163, respectively. To test inhibition of TWEAK binding to CD163 by anti-CD163 Abs, CD163 protein was coated, blocked with PBS containing 2% BSA, and incubated with anti-CD163 Abs (clones Ki-M8, GHI/61, and 10D6) at 200 ng/ml for 30 min at room temperature. Control IgGs were used at same conditions. Then, rHuTWEAK was added onto the wells for 2 h and developed with anti-TWEAK (1/1,000 dilution). TWEAK binding to CD163 was evaluated by preincubation of rHuTWEAK with CWWDGWSFC or control peptides added at 0, 3, 6, 10, 30, 60, 100, and 300 μM).

PBMCs and monocyte purification

Fresh human PBMCs were isolated from 50–60 ml of buffy coat from normal volunteer blood donors (Gulf Coast Blood Bank, Houston, TX) by using Ficoll-Paque (Pharmacia Biotech) density gradients. Monocytes were isolated from the PBMC population by positive selection using a CD14-positive magnetic bead separation system (Miltenyi Biotec). Cells were used immediately for flow cytometry or cultured in AIM-V medium (Invitrogen Life Technologies) for other assays.

Flow cytometric analysis for CD163 expression and other SRCR expression

CD14-positive monocytes were incubated on ice for 1 h with PE-labeled mouse anti-human-CD163 (clone GHI/61), FITC-conjugated anti-human

[5] Abbreviations used in this paper: SRCR, scavenger receptor cystein rich; CHO, Chinese hamster ovary; Cy3, cyanine 3; EGF, epidermal growth factor; Fn14, fibroblast growth factor inducible 14; Hb, hemoglobin; Hp, haptoglobin; rHuTWEAK, recombinant human TWEAK; ^{125}I-rHuTWEAK, ^{125}I-labeled rHuTWEAK; TU, transforming unit.

Sequences	n=158	(Frequency%)
RWADDGF	8	(5.1)
FWADDGF	2	(1.3)
YWADDGF	1	(0.6)
YWGDDGF	3	(1.9)
SWGDDGY	4	(2.5)
RWGDDGY	1	(0.6)
FWGDDGW	1	(0.6)
RWEDDGF	4	(2.5)
VWEDDGF	3	(1.9)
WWFDDGY	4	(2.5)
LWTDDGY	2	(1.3)
HWTDDGY	2	(1.3)
EWRDDGW	1	(0.6)
WRDDGWS	1	(0.6)
FWRDDGW	1	(0.6)
WWRDDGF	1	(0.6)
LWYDDGW	2	(1.3)
FWVDDGY	1	(0.6)
RWQDDGL	1	(0.6)
W-DDGWWD	2	(1.3)
W-DDGWSF	1	(0.6)
W-DDGFYP	2	(1.3)
WFDDGF	1	(0.6)
WEED-LDV	6	(3.8)
W-EDTLDW	2	(1.3)
W-EDWLDK	1	(0.6)
W-EDTLDH	1	(0.6)
W-EDTLDM	1	(0.6)
WDWDGWT	1	(0.6)
WDWDGWP	1	(0.6)
SWDGWVI	1	(0.6)
FWDGWGT	2	(1.3)
YWDGWGT	1	(0.6)
FWDGWGV	1	(0.6)
FWDGWDL	2	(1.3)
FWDGWSL	1	(0.6)
YWDGWLD	2	(1.3)
DPHWDGW	1	(0.6)
YPHWDGW	1	(0.6)
WLEWDGW	1	(0.6)
WGGWDGW	1	(0.6)
WWGWDGW	1	(0.6)
NFWDGLG	2	(1.3)
LHWDGYG	1	(0.6)
WPEWDGL	1	(0.6)
W-EDGWYA	2	(1.3)
W-EDGIFF	2	(1.3)
W-EDGICL	1	(0.6)
W-EDGLWQ	1	(0.6)
W-EDGWYA	2	(1.3)
RWLEDGY	1	(0.6)
RWKEDGK	1	(0.6)
SWGEDGY	1	(0.6)
YWGEDGY	1	(0.6)
EFREDGW	1	(0.6)
FYREDGW	1	(0.6)
Total	95	(60.0)
Others	63	(40.0)

FIGURE 2. Phage sequences isolated by selection on TWEAK-GST.

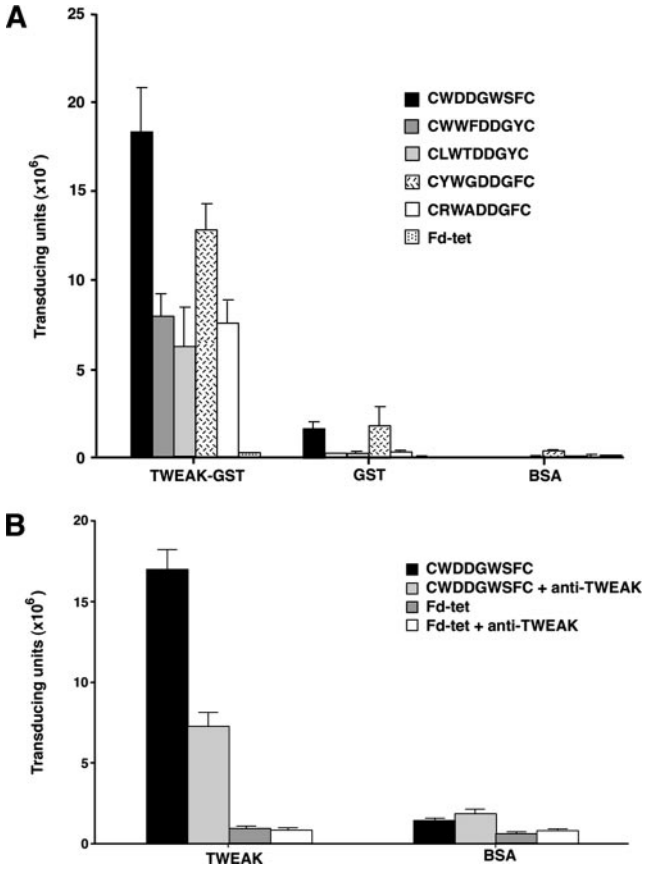

FIGURE 3. Binding of selected peptides to TWEAK. *A*, Phage clones displaying individual peptide sequences or negative control phage (insertless Fd-tet) were incubated on microtiter wells (10^9 TU per well) coated with TWEAK-GST, GST, or BSA. *B*, Binding inhibition of CWDDGWSFC phage to TWEAK by an anti-TWEAK polyclonal Ab. Microtiter wells coated with rHuTWEAK were incubated with goat anti-human anti-TWEAK Ab. CWD DGWSFC phage was added (10^9 TU) and incubated for additional 2 h. Shown is mean ± SEM of a representative experiment. The experiments were repeated three times with similar results.

CD14, PE-anti-mouse and human Fn14/TweakR (PE-ITEM-4), or the respective isotype controls (PE-mIgG1/k, PE-mIgG2b/k, and FITC-mIgG2a). For inhibition assays, monocytes were incubated with either 200 ng/ml rHuTWEAK or PBS at 4°C. Cells were then washed and left not fixed or fixed in PBS containing 2% paraformaldehyde and then treated with PE-anti-human CD163. After processing, cells were fixed in PBS containing 2% paraformaldehyde and subjected to flow cytometry analysis by using a FACScan flow cytometer (BD Biosciences) with CellQuest software. To analyze the cell surface expression of SRCR or Fn14 on the CHO cells, CD163-, CD5-, CD6-, or Fn14-transient transfectant or wild-type CHO cells (10^6 cells/200 μl) were incubated with a mouse mAb against CD163, CD5, CD6, Fn14, or control mouse IgG at 4°C for 1 h followed by FITC-labeled secondary Ab to at 4°C for 30 min and then subjected to flow cytometry analysis.

Confocal immunofluorescence microscopy

CD14-positive monocytes plated overnight in the LabTek II chamber slide system (2.5×10^5 cells/well) were incubated with 200 ng/ml rHuTWEAK or PBS for 30 min at 4°C to prevent or minimize receptor internalization. After three washes with ice-cold PBS, cells were incubated with an polyclonal goat-anti-human TWEAK Ab. A Cy3-conjugated AffiniPure $F(ab')_2$ donkey anti-goat IgG (H+L) was used as a secondary Ab. Fixed slides mounted with fluorescent mounting medium (DakoCytomation) were examined in a confocal microscope (Olympus FV500).

Monocyte activation assay

Monocyte activation was measured by quantifying the ability of cells to metabolize the tetrazolium salt WST-1 to formazan (Roche) at 37°C (34). CD14-positive monocytes were plated in 96-well plates (30,000 cells/well) and incubated for 42 h with or without dexamethasone (10 ng/ml; Sigma-Aldrich), rHuTWEAK (1 μg/ml) and anti-CD163 GHI/61 (1.25 μg/ml). WST-1 was added for 4 h after finishing the incubation period. Absorbance was determined at 440 nm in an optical reader (Bio-Tek Instruments).

TWEAK-depleted supernatants

Freshly isolated human monocytes preincubated with or without anti-CD163 Ab for 30 min were treated with increasing concentrations of

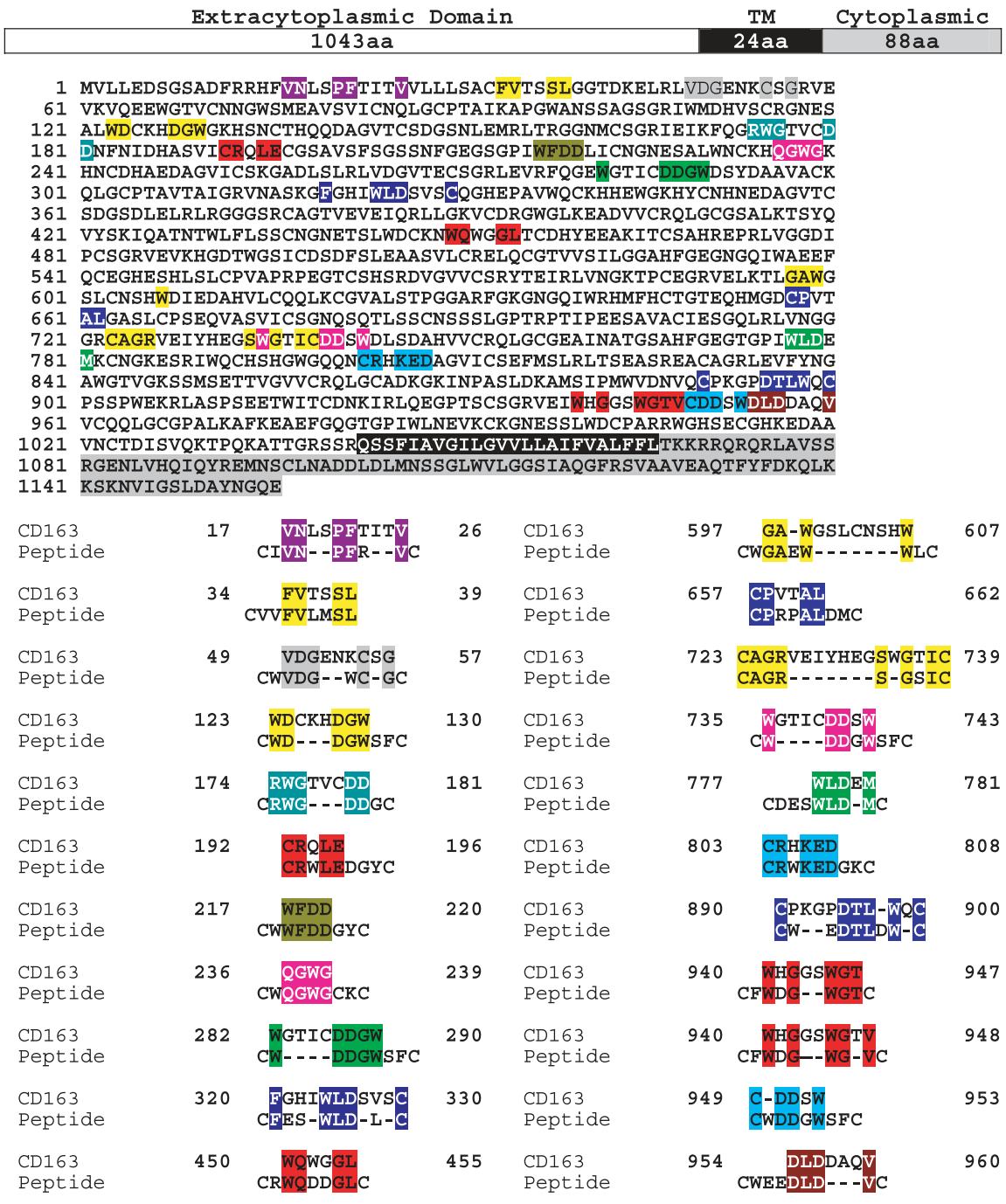

FIGURE 4. Alignment of peptide sequences isolated from in vitro selection on TWEAK and CD163 SRCR protein.

rHuTWEAK (0, 10, 50, and 100 ng/ml) for 1 h at 4°C or 24 h at 37°C. After that, monocytes were decanted by centrifugation and supernatants were frozen at −80°C until additional experiments were performed. Additional time points were also obtained by treating monocytes for 30 min and 2 h (data not shown). Alternatively, rHuTWEAK was preincubated with CWDDGWSFC or the control peptide (30 and 100 μM) for 30 min before admixture into the monocyte culture.

Cell viability on TWEAK-sensitive cells

Human colon adenocarcinoma HT-29 cells were obtained from American Type Culture Collection and cultured with modified RPMI 1640 containing 10% FBS, 100 μg/ml streptomycin and penicillin, and 2 mM glutamine. Cells were plated in 96-microtiter well plates (20,000 cells/well) and pretreated with IFN-γ (20 ng/ml; R&D Systems) for 12 h. Then, cells were incubated with or without rHuTWEAK as indicated. After 36 h of culture, cell proliferation was assessed. To evaluate the ability of monocytes to deplete TWEAK from the medium, supernatants from monocyte cultures (as described above) were added to IFN-γ-treated HT29 cells and a cell viability assay was performed under the same conditions. To rule out the possibility of TWEAK degradation by monocyte-secreted proteins, parallel experiments were performed by using a conditioned medium of cultured monocytes incubated with or without same amounts of rHuTWEAK, and a cell viability assay was conducted (data not shown).

Transient expression of huCD163 in CHO cells

The open reading frames of CD5 and CD6 were directionally cloned in the eukaryotic vector pCMV and confirmed by DNA sequencing. Plasmids

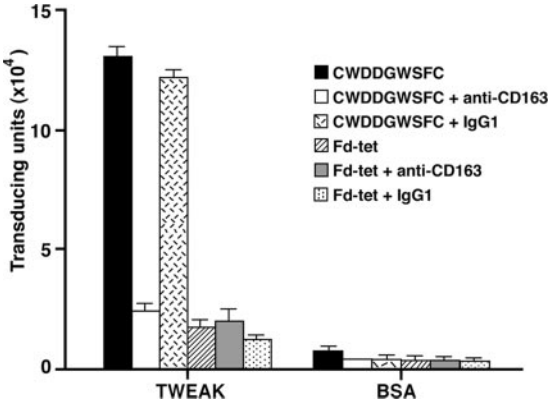

FIGURE 5. Binding inhibition of CWDDGWSFC phage to TWEAK by an anti-human CD163 mAb. Either CWDDGWSFC phage or insertless phage at 10^9 TU was incubated with 10 μg/ml anti-human CD163 mouse mAb. A control isotype Ab was used as a negative control. Afterward, each solution was transferred to microtiter wells coated with rHuTWEAK or BSA and incubated for an additional 2 h. Shown is mean ± SEM of a representative experiment. Three experiments were performed with similar results.

were prepared by using the GenElute HP Endotoxin-free kit (Sigma-Aldrich) for cell transfection. CHO cells were maintained with RPMI 1640 medium containing 5% FBS and no antibiotics. Cells were seeded at 30% confluence into 24-well plates and transfected at a ratio of 6 μl of FuGENE per 1 μg of DNA with the plasmids encoding human SRCR or Fn14. Twenty-four hours after transfection, the cells were washed once with PBS and fresh medium containing the adequate ligands was added. Cell surface expression of the transfectants was analyzed by the flow assay cytometry described above.

Preparation of ^{125}I-rHuTWEAK and Hp-Hb complexes

^{125}I-rHuTWEAK was prepared by using an Iodogen coating tube (Pierce) as described (35). Briefly, 200 μg of rHuTWEAK was incubated with Na^{125}I (0.925 MBq) in 200 μl of 0.5 M sodium phosphate buffer (pH 7.4) for 20 min at 37°C, and then dialyzed against PBS. Complexes were prepared by mixing equimolar concentrations of Hb and Hp in PBS as described (30).

Cellular binding of ^{125}I-rHuTWEAK by CD163-CHO cells

Cellular binding of ^{125}I-rHuTWEAK was performed in accordance to that of advanced glycated end product-modified BSA by CD36 as described previously (35). For cell binding assay, cells were incubated with 100 ng/ml ^{125}I-rHuTWEAK at 4°C in RPMI 1640 containing 3% BSA for 3 h. After incubation, cells were washed three times with ice-cold PBS and lysed with 0.1 M NaOH. Radioactivity and protein concentration of whole cell lysates were determined by using a gamma well counter and a BCA protein assay kit (Pierce). For inhibition assays, cells were also incubated with or without the indicated ligands (1 or 10 μg/ml rHuTWEAK or Hp-Hb complexes, 100 μM CWDDGWSFC or control peptide CRKFRDEATC, and 10 μg/ml AM-3K anti-CD163 Ab or control mouse IgG).

Results

Identification of TWEAK-binding peptides

We isolated TWEAK-binding peptides by screening a CX_7C phage library (22, 23) on a recombinant TWEAK-GST fusion protein (Fig. 1). A marked enrichment in phage binding to TWEAK-GST relative to the negative controls GST (30-fold) or BSA (2,000-fold) was observed after three rounds of selection.

Analysis of peptide sequences and specificity of binding to TWEAK

Forty-nine of 158 (31%) of clones obtained from the screening displayed a WXDDG motif or some variation of it; X was preferentially a nonpolar or polar uncharged residue (Fig. 2). Phage clones displaying the peptide sequences CWDDGWSFC, CLWTD DGYC, CYWGDDGFC, CWWFDDGYC, and CRWADDGFC were used for additional experiments. Such sequences were chosen because they were enriched or because they showed homology to candidate binding proteins. The relative binding of these peptide sequences to TWEAK in regard to negative controls was then individually determined (Fig. 3*A*). Selected peptides showed an enhanced binding to TWEAK relative to GST (mean 26-fold; range 7- to 48-fold). Phage clones displaying the peptide CWDDGWSFC had the

A. Alignment of human TWEAK (NP_003800) with human haptoglobin (NP_005134)

```
TWEAK       117   PGQDGAQAG-VDGTVSGW-EEARINSSSPLRY 146
Haptoglobin 268   PSKDYAEVGRV-GYVSGWGRNANFKFTDHLKY 298
```

B. Alignment of human TWEAK (NP_003800) with hemoglobin b-chain (P02023)

```
TWEAK              154   IVTRAGLYY-LYCQVHFDE-G   172
Hemoglobin β-chain  11   AVT-A-LWGKVN--V--DEVG    25
```

C. Human TWEAK protein sequence matching with haptoglobin and hemoglobin residues

```
TWEAK isoform 1 (NP_003800)

  1 MAARRSQRRRGRRGEPGTALLVPLALGLGLALACLGLLLAVVSLGSRASLSAQEPAQEEL
 61 VAEEDQDPSELNPQTEESQDPAPFLNRLVRPRRSAPKGRKTRARRAIAAHYEVHPRPGQD
121 GAQAGVDGTVSGWEEARINSSSPLRYNRQIGEFIVTRAGLYYLYCQVHFDEGKAVYLKLD
181 LLVDGVLALRCLEEFSATAASSLGPQLRLCQVSGLLALRPGSSLRIRTLPWAHLKAAPFL
241 TYFGLFQVH

S Cleavage site
PGQ...LRY =Hp homologue region
IVT...DEG =Hb homologue region
```

D. Alignment of phage clones that bind TWEAK with the heavy chain CDR3 of a mAb raised against the Hp-Hb complex

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CDR3          EDTTDWY-FDV
Clone 15    CWEDTLDWC
Clone 17          CWYLFDEPC
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FIGURE 6. Protein sequence alignment of TWEAK and haptoglobin and hemoglobin.

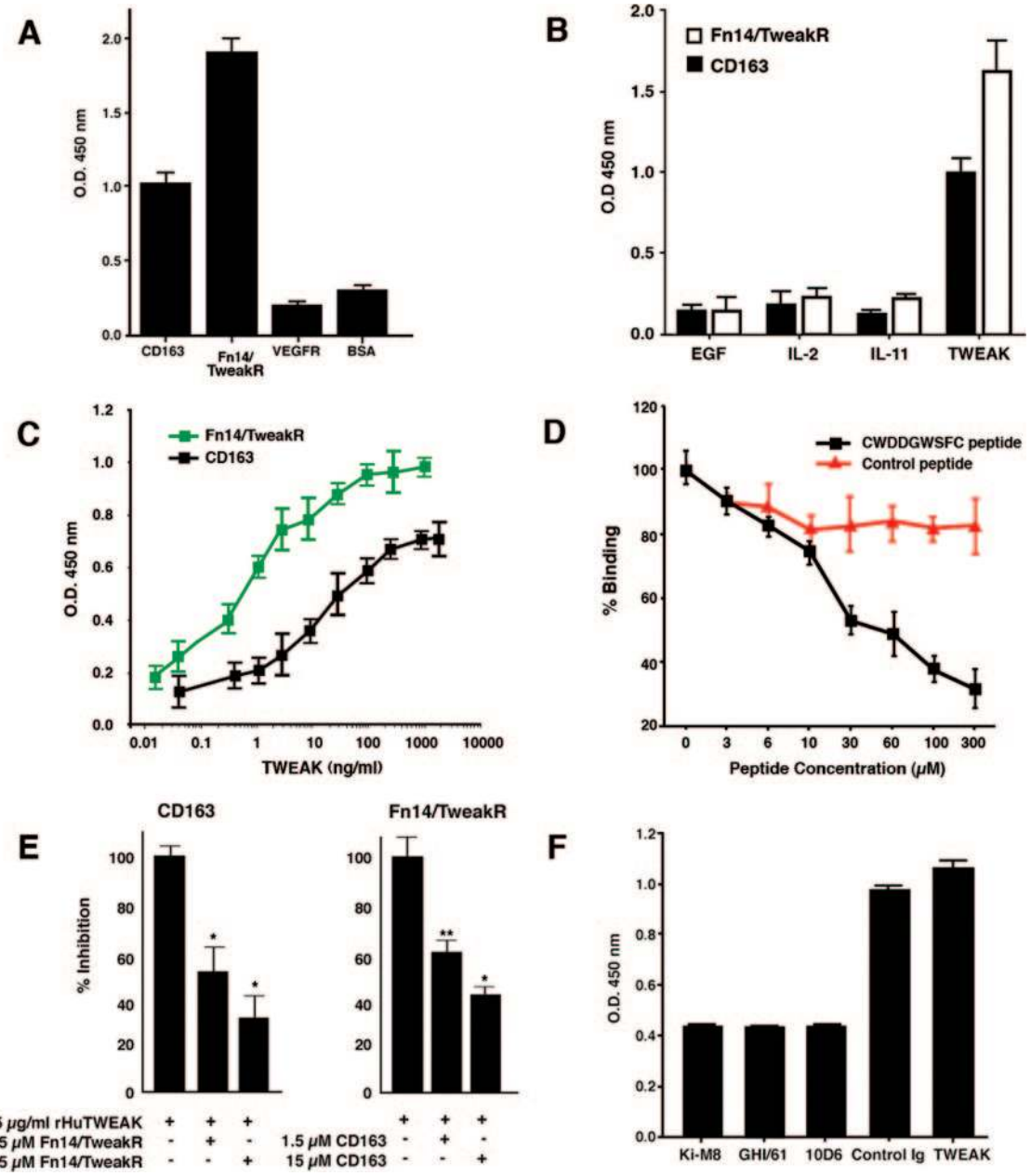

FIGURE 7. Dose-dependent and inhibitable binding of TWEAK to CD163. *A*, rHuTWEAK binding to CD163. TWEAK was added (50 μl of 500 ng/ml) to the CD163-coated well. Vascular endothelial growth factor receptor (VEGFR) and BSA served as coated negative controls. Bound protein was measured by ELISA. *B*, Interactions of soluble cytokines with CD163. CD163 (filled bars) or Fn14/TweakR (open bars) proteins (1 μg/ml) were coated onto 96-microtiter well plates and incubated with 500 ng/ml EGF or IL-11 or 100 ng/ml IL-2. Cytokine binding was detected with anti-EGF, anti-IL-11, and anti-IL-2 Abs. *C*, Dose-dependent binding of TWEAK to CD163. CD163 was coated onto a 96-microtiter well plate and incubated with increasing amounts of rHuTWEAK. *D*, Dose-dependent inhibition of TWEAK-CD163 interaction by the CWDDGWSFC peptide was measured by ELISA. *E*, Inhibition of TWEAK-receptor binding by soluble receptor. rHuTWEAK preincubated with Fn14/TweakR for 30 min at room temperature was added to CD163 coated plate (*left panel*). rHuTWEAK preincubated with CD163 for 30 min at room temperature was added to Fn14/TweakR coated plate (right panel) (*t* test; *, $p < 0.001$; **, $p < 0.004$). *F*, Inhibition of TWEAK-CD163 interaction by CD163 Abs. CD163 protein was coated onto 96-microtiter well plates. Anti-CD163 Abs (clones Ki-M8, GHI/61, and 10D6) were added into wells for 30 min at room temperature before adding rHuTWEAK. Isotype IgG was used as a negative control. Coated rHuTWEAK with no Abs added served as a positive control. Results from two representative experiments are shown.

highest relative binding to TWEAK (10-fold for GST and 900-fold for BSA) and bound over 800-fold more than binding of the insertless control phage. Similar results were also obtained with the purified rHuTWEAK protein. To evaluate the specificity of the interaction between CWDDGWSFC and TWEAK, we tested whether CWDDGWSFC-phage binding to TWEAK was inhibited when immobilized rHuTWEAK was preincubated with an anti-TWEAK Ab. CWDDGWSFC-displaying phage binding (but not binding of the control phage) was inhibited (Fig. 3*B*). Together, these data indicate that the CWDDGWSFC interacts specifically with TWEAK.

Mapping of peptide sequences to CD163

Peptide sequences obtained from the phage selection ($n = 158$) were analyzed for similarity to known human proteins by using the NCBI BLAST software. Of these sequences, 41 (26%) matched motifs found within the CD163 protein (NCBI protein

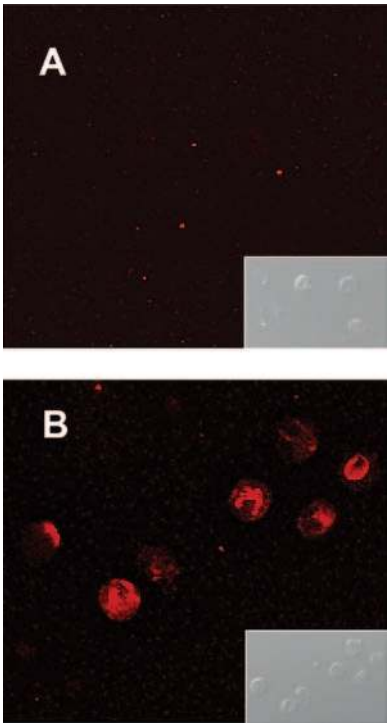

FIGURE 8. In situ detection of TWEAK binding to a subset of CD14-positive monocytes. Human CD14-positive monocytes plated overnight in AIM-V medium were incubated with rHuTWEAK or PBS for 30 min at 4°C. After washing, cells were fixed and incubated with goat anti-human TWEAK and a secondary Ab conjugated to Cy3. Cells were examined under a confocal microscope. *A*, PBS. *B*, rHuTWEAK. *Insets*, The corresponding contrast phase images. Original magnification, ×400.

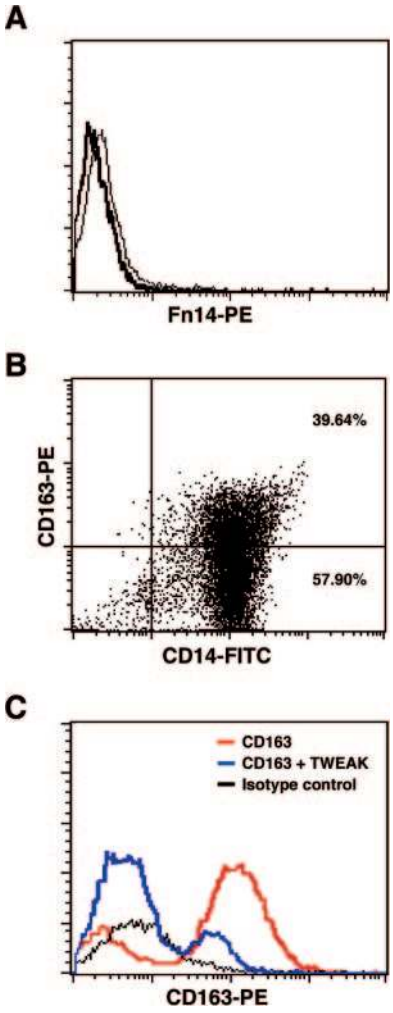

FIGURE 9. Expression of CD14, CD163, and Fn14/TweakR surface molecules on freshly isolated CD14-positive monocytes. CD14-positive monocytes were labeled with anti-CD14-FITC, anti-CD163-PE, an Ab against Fn14/TweakR, or their respective control isotype Abs. *A*, Cells incubated with anti Fn14/TweakR-PE or control isotype. *B*, Cells incubated with anti-human-CD14-FITC and anti-human-CD163-PE. *C*, Cells were preincubated with 200 ng/ml rHuTWEAK (blue) or PBS (red) for 30 min at 4°C and then incubated with anti-human CD163-PE. Incubation with isotype control (black) was included. A representative experiment is shown.

database accession no. NP_004235). CD163 is a member of the SRCR superfamily and is expressed on the human monocyte and macrophage lineage. An alignment of a subset of peptide sequences isolated from panning on TWEAK with the protein sequence of CD163 was assembled (Fig. 4). Because the CWDDGWSFC peptide aligned to different sites of the CD163 sequence (data not shown), we estimate a total of 44 putative interacting sites between TWEAK and CD163. As predicted, all sites located to the extracellular domain containing the SRCR of the CD163 protein.

CWDDGWSFC mimics CD163

Based on these observations, we hypothesized that the selected TWEAK-binding phage clones mimic CD163. To test this hypothesis, we evaluated the specific recognition of the CWDDGWSFC-phage by an anti-CD163 mAb raised against the N-terminal domain of the protein (which includes the cysteine-rich domain). We next performed a binding experiment in vitro in which the CWDDGWSFC-phage or the control phage was preincubated with anti-CD163 mAb or an isotype control. The anti-CD163 mAb inhibited the binding of the CWDDGWSFC-phage to immobilized rHuTWEAK, whereas the isotype control did not exert any detectable blocking effect (Fig. 5). This result suggests that the CWDDGWSFC peptide mimics, at least partially, the epitope recognized by the anti-CD163 mAb.

TWEAK exhibits similarity to a native CD163 ligand

The SRCR CD163 was identified as a specific Hb scavenger receptor that mediates the uptake of Hb in complex with Hp (i.e., the Hp-Hb complex; Refs. 29 and 36). A search for similarities in the protein sequences of TWEAK, Hp, and Hb led to several observations. First, TWEAK (NCBI protein database accession no. NP_003800) and Hp (NCBI protein database accession no. NP_005134) each contained a region consisting of a 31-residue motif with 13 identical amino acids and six amino acids with the same hydropathic profile (Fig. 6*A*). This region represents 19% of secreted TWEAK (residues 95–249) and 12% of the Hp β-chain (residues 162–406). We also observed that the residues 11–25 in the Hb β-chain (NCBI protein database accession no. P02023), which are also implicated in Hp-Hb complex formation (37), matched a region comprising amino acids 154–172 in TWEAK (Fig. 6*B*). This segment in TWEAK is separated by only seven residues from the domain homologous to Hp (Fig. 6*C*). Finally, the CDR3 of a Fab Ab (10 residues; sequence EDTTDWYFDV) that blocks the binding of Hp-Hb complex to CD163 (38) revealed >50% homology with the CWEDTLDWC peptide (Fig. 4) and ~40% homology with the CWYLFDEPC peptide (Figs. 6*D* and 2) of the screening.

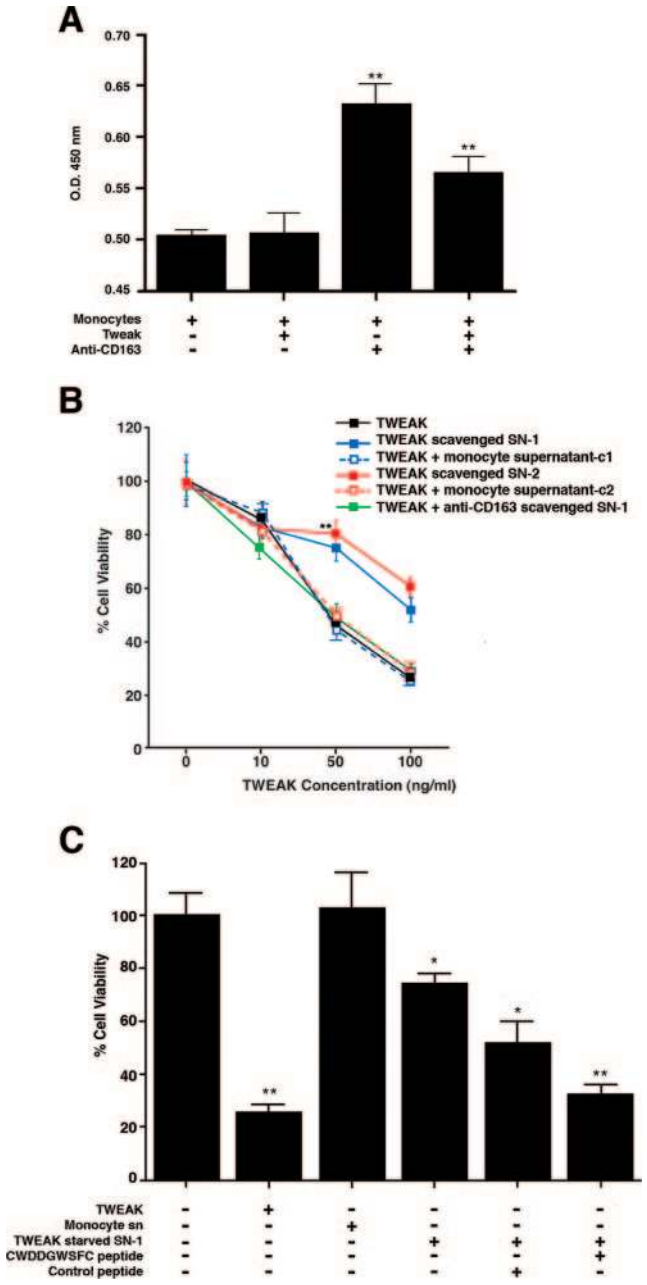

FIGURE 10. Interaction between TWEAK and CD163 in human monocytes. *A*, Effects of anti-CD163 mAb and TWEAK on monocyte activation. CD14-positive monocytes were plated in 96-well plates (30,000 cells/well). After 4 h of plating, rHuTWEAK (1 μg/ml) and anti-CD163 mAb GHI/61 (1.25 μg/ml) were added. Cells were incubated for 42 h and pulsed for 4 h with WST-1. Absorbances were measured at 450 nm. An isotype control had no detectable effect (data not shown). Shown are the means ± SEM of a representative experiment (*t* test; *, $p < 0.005$; **, $p < 0.001$). *B*, TWEAK-mediated programmed cell death can be inhibited by preincubation of TWEAK with monocytes, but not in the presence of an anti-CD163 Ab. HT-29 cells (20,000 cells/well) in the presence of IFN-γ (20 ng/ml; black line) were cultured with the indicated concentrations of rHuTWEAK or with supernatants from TWEAK-treated (1 h) monocytes (blue line, SN1 (supernatant from freshly isolated monocytes); red line, SN-2 (supernatant from 24 h cultured monocytes)). After 36 h in culture, cell viability was assessed by the WST-1 assay. Data are represented as the mean ± SEM of triplicate samples. Similar results were obtained in three independent experiments. *C*, Inhibition of TWEAK-mediated programmed cell death by the CWDDGWSFC peptide. IFN-γ treated HT29 cells were cultured with supernatants from freshly isolated monocytes containing 100 ng/ml rHuTWEAK coincubated with 100 μM CWDDGWSFC peptide or control peptide. Culture medium, SN-1 supernatant and medium with 100 ng/ml rHuTWEAK were used as controls (*t* test; *, $p < 0.005$; **, $p < 0.001$).

TWEAK binds to CD163

To support our hypothesis, we evaluated the binding of rHu TWEAK to CD163. We show that TWEAK binds specifically to CD163 in a dose-dependent manner (Fig. 7, *A* and *C*). As controls, binding to Fn14/TweakR but not to an unrelated receptor (variable endothelial growth factor receptor) or BSA are shown (Fig. 7*A*). Human EGF, IL-2, and IL-11 also failed to bind to CD163 (Fig. 7*B*). Next, we showed that TWEAK binding to CD163 is inhibited by a CWDDGWSFC synthetic peptide in a dose-dependent manner; a control peptide with an unrelated sequence had no effect (Fig. 7*D*). We sought to determine whether the addition of the soluble receptor would interfere with the TWEAK binding. Soluble CD163 preincubated with rHuTWEAK was able to inhibit the binding to Fn14/TweakR (Fig. 7*E*, *left panel*). In contrast, Fn14/TweakR preincubated with rHuTWEAK also inhibited TWEAK binding to CD163 (Fig. 7*E*, *right panel*) suggesting a competition between both receptors. Recently, Madsen et al. (39) described ligand-binding properties of the membrane-associated CD163 protein and identified the SRCR domains involved in Hp-Hb and Ab binding. Thus, we next evaluated whether preincubation with three different clones of anti-CD163 Abs (Ki-M8, GHI-61, and 10D6) could inhibit TWEAK binding to CD163. Consistent with our findings, all the three Abs inhibited TWEAK binding to CD163 (Fig. 7*F*).

Fn14/TweakR-negative/CD163-positive human monocytes specifically bind TWEAK

Because TWEAK is not present at detectable levels on the surface of freshly isolated monocytes (9), we asked whether monocytes would bind TWEAK. CD14-positive monocytes, selected because this subset of cells displays the highest expression of CD163 among monocytes (26), did not show expression of TWEAK on the surface after 18 h in culture (Fig. 8*A*); however, we detected TWEAK binding at the cell surface after incubation with rHuTWEAK (Fig. 8*B*). To further investigate the protein responsible for TWEAK binding in CD14-positive human monocytes, we first evaluated the expression of Fn14/TweakR and that of CD163. Flow cytometry revealed no expression of the recently identified receptor of TWEAK (Fn14/TweakR; Refs. 16 and 17), suggesting the presence of another TWEAK-binding protein on CD14-positive human monocytes. In contrast and in agreement with previous reports (26), we found a high expression of CD163 in 40% of CD14-positive monocytes, (Fig. 9, *A* and *B*). To determine whether TWEAK and CD163 interact specifically at the surface of human monocytes, we analyzed the expression of CD163 after the preincubation of freshly isolated CD14-positive cells with TWEAK. We found that the detection of CD163 with an anti-CD163 mAb was inhibited in the presence of TWEAK, but no effect was noted when the cells were preincubated with PBS alone (Fig. 9*C*). Collectively, these data are consistent with a specific interaction between TWEAK and CD163 on the cell surface of Fn14/TweakR-negative human monocytes.

Monocyte activation induced by an anti-CD163 Ab is inhibited by TWEAK

We evaluated whether TWEAK and CD163 can interact functionally. Cross-linking of the CD163 receptor by an anti-CD163

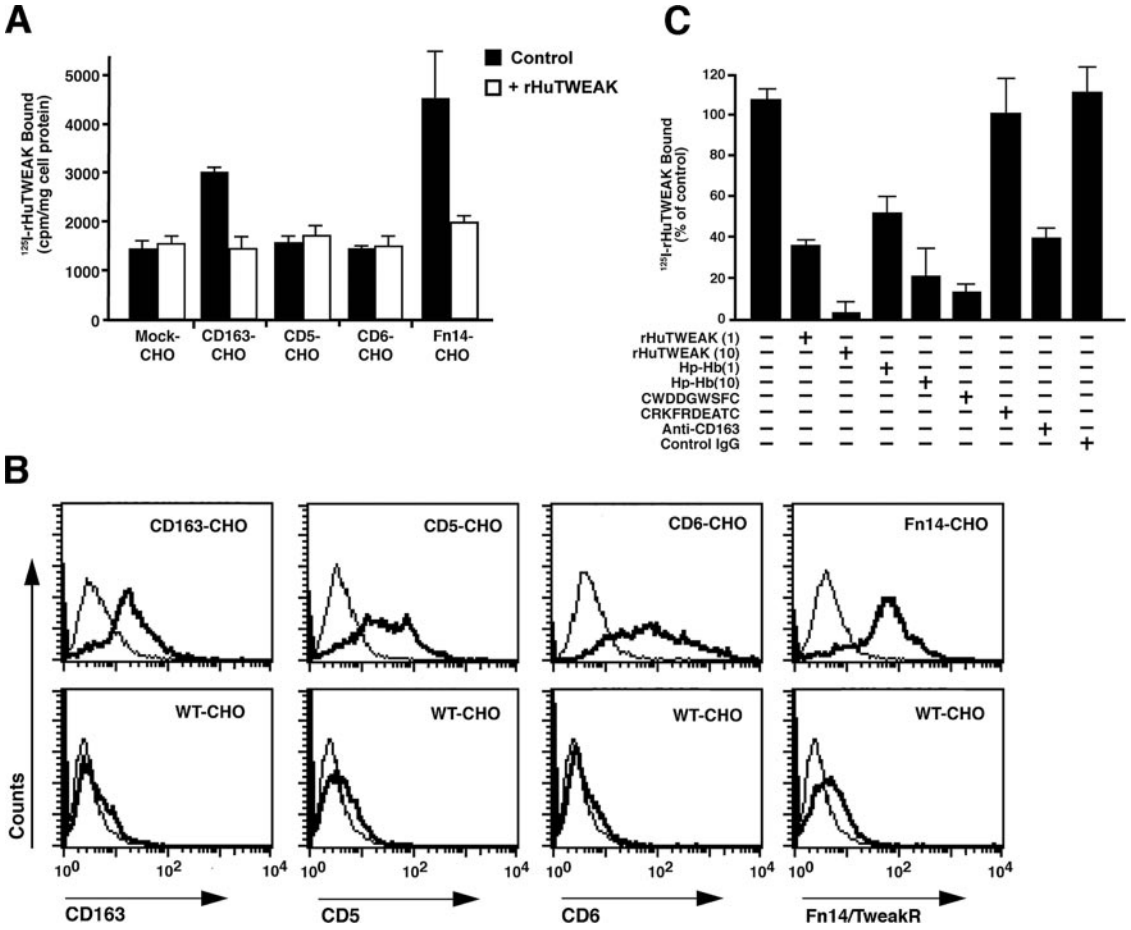

FIGURE 11. Cellular binding of ^{125}I-rHuTWEAK to the CD163-expressed CHO cells. *A*, CHO cells were transiently transfected with or without plasmids encoding human SRCR (CD163, CD5, and CD6) or Fn14/TweakR. The CHO cells transiently expressing SRCR, Fn14/TweakR, or mock-transfectant control were incubated at 4°C for 3 h with 100 ng/ml ^{125}I-rHuTWEAK in the presence (open bar) or absence (filled bar) of an excess amount of unlabeled rHuTWEAK (10 μg/ml). After washing, cells were lysed with 0.1 M NaOH and the radioactivity of the whole cell lysates was determined. *B*, Cell surface expression by flow cytometry of CD163, CD5, CD6, and Fn14/TweakR on the CHO cells. CHO cells transiently transfected with CD163, CD5, CD6, or Fn14/TweakR (*upper histograms*) and wild-type (WT) CHO cells (*lower histograms*) were incubated with each mouse mAb (bold line), or control mouse IgG (standard line). *C*, Effects of the CD163 ligands on the binding of the ^{125}I-rHuTWEAK. CD163-expressed CHO cells were incubated at 4°C for 3 h with ^{125}I-rHuTWEAK (100 ng/ml) in the presence or absence of various ligands (1 or 10 μg/ml rHuTWEAK or Hb-Hp complexes, 100 μM CWDDGWSFC or control peptide CRKFRDEATC) or 10 μg/ml Abs (anti-CD163 AM-3K or control mouse IgG). An amount of ^{125}I-rHuTWEAK bound to the CD163 was determined. Specific amounts were plotted after subtracting nonspecific binding by mock transfectants. Values represent mean ± SD (n = 3).

mAb induces signaling and macrophage activation (28). There are two technical considerations that may well be relevant to interpreting this set of experiments. First, culturing normal monocytes results in an ~60% decrease in cell number after 48 h, with most of those cells being lost during the first 6 h of culture (40). Second, the ability of viable cells to metabolize tetrazolium salts (indicated as WST-1 reduction), generally used as a quantitative measure of cell number, can also reflect cell activation from an increase in the overall activity of mitochondrial dehydrogenases (34, 41).

We sought to determine whether incubation with the anti-CD163 Ab GHI/61 activated CD14-positive human monocytes and whether coincubation with TWEAK changed such an effect. The ability of cells to metabolize tetrazolium salts was used as a cell activation marker to evaluate differences in cell viability (34). Activation was higher for monocytes incubated with anti-CD163 Ab relative to buffer (Fig. 10*A*) or isotype control (data not shown). This effect was abrogated by the addition of rHuTWEAK. In comparison to control cultures, monocytes incubated with rHu TWEAK alone showed no difference in activation level. The addition of dexamethasone, which up-regulates CD163 (24), did not change the results. Thus, TWEAK appears to inhibit anti-CD163-Ab-induced activation by competing for the same or perhaps a close overlapping binding site (Fig. 10*A*).

TWEAK-mediated programmed cell death can be inhibited by preincubation with monocytes

TWEAK-induced programmed cell death in several tumor cell lines has been extensively characterized. In particular, Nakayama et al. (12) described the mechanisms mediating the apoptosis of IFN-γ-treated HT-29 cells. Based on the results described above, we hypothesized that CD163, a scavenger receptor, might be acting as a receptor "decoy" for the ligand TWEAK. To test this hypothesis experimentally, we evaluated whether human monocytes (either freshly isolated or after plating for 24 h) could bind and functionally sequester the rHuTWEAK added to the culture medium. Indeed, we show that the ability of TWEAK to induce apoptosis is abrogated from TWEAK-containing monocyte supernatants compared with controls (Fig. 10*B*). To rule out the possibility that TWEAK was actually being degraded or consumed by

monocyte-secreted proteins, we incubated monocyte-conditioned medium (collected at 30 min and at 1 and 2 h) with the same amounts of TWEAK used above. No differences were observed compared with the curve for untreated TWEAK (data not shown). Moreover, TWEAK-mediated programmed cell death was observed when monocytes were treated with an anti-CD163 Ab (Fig. 10*B*) or by preincubating TWEAK with the CWDDGWSFC peptide (Fig. 10*C*).

Cellular binding of ^{125}I-rHuTWEAK to CD163

To examine the cellular interaction of CD163 with TWEAK, we determined the binding of ^{125}I-rHuTWEAK by SRCR (CD163, CD5, and CD6) or TWEAK receptor Fn14 compared with mock-transfected CHO cells. Fig. 11*A* shows that CHO cells transiently expressing CD163 exhibited the specific binding of ^{125}I-rHuTWEAK but that mock-transfected CHO cells did not. As positive control, ^{125}I-rHuTWEAK bound to the Fn14-expressed cells. In contrast, CHO cells expressing CD5 or CD6 have no specific binding activity. These results indicate that TWEAK is specifically recognized by CD163 as well as Fn14, but not by CD5 and CD6. Cell membrane expression of CD163, CD5, CD6, and Fn14/TweakR on CHO cells was demonstrated by flow cytometry (Fig. 11*B*).

Next, we examined the effects of various ligands and Abs on the binding of TWEAK to the CHO cells transiently expressing CD163. ^{125}I-rHuTWEAK binding to the CD163 was inhibited completely by unlabeled rHuTWEAK in a dose-dependent manner and almost inhibited by an excess of Hb-Hp complexes (Fig. 11*C*). In addition, the CD163-like peptide CWDDGWSFC and the anti-CD163 mAb AK-3K (30) also decreased this activity by 15 and 37%, respectively. By contrast, two controls (CRKFRDEATC peptide and mouse IgG) had no effects. Furthermore, CD163-CHO cells mediated the specific degradation of ^{125}I-rHuTWEAK, but mock-transfected or cells expressing CD5 or CD6 had no such activity (data not shown). These results indicate that the TWEAK binding site(s) within CD163 shares their recognition site(s) with that of Hb-Hp complexes, suggesting that CD163 serves as a scavenger receptor for both ligands.

Discussion

Several studies based on TWEAK-induced migration, proliferation, and angiogenesis in endothelial cells and apoptosis in tumor cells have long suggested a role for the TWEAK receptor Fn14/TweakR (1–3, 17). However, the wide range of biological effects in cells deficient for that receptor raises the possibility that cells may express other TWEAK receptors. First, we screened a combinatorial library to find TWEAK-binding peptides and found motifs similar to that of CD163. Moreover, we validated functional protein-protein interactions between TWEAK and CD163. Finally, we show that the binding is specific. The findings reported here support the candidacy of the cell surface protein CD163 as a TWEAK-binding protein and a possible alternative TWEAK receptor in Fn14/TweakR-deficient cells.

After the selection of a random peptide library on immobilized TWEAK, marked enrichment was obtained. Upon DNA sequencing of the selected phage clones, it became apparent that many of the peptides displayed contained the predominant motif WXDDG or its permutations (flanked by cysteine residues engineered into the libraries). Of note, we have previously identified similar peptide motifs as structural sites in certain integrins (19) and aminopeptidases (22), indicating that this motif is both conserved in several protein families and functional in ligand-receptor binding interactions.

In this study, sequence homology analysis of the predominant motifs recovered from the selection (Figs. 2 and 4) suggest that the peptides mimic the structure of CD163, a member of the class B SRCR restricted to the monocyte/macrophage lineage. SRCR domains are widely found in cell surface molecules where they are thought to mediate ligand binding (39); in particular, the CD163 sequence has nine extracellular cystein-rich domains. Indeed, repeated units of cysteine clusters also define the TNFR superfamily (42). The presence of this motif suggested the possibility that such domains may mediate a putative protein interaction between TWEAK and CD163. The crystal structure of another member of the SRCR family, the glycoprotein M2BP (Mac-2 binding protein; M2BP) (43), has been considered a general template for the cysteine-rich domains in proteins of this ancient superfamily (43, 44), including CD163. It consists of a curved six-stranded β-sheet cradling an α-helix. When TWEAK-binding peptides were aligned with the CD163 sequence, the putative sites of interaction were identified in eight of the nine cysteine-rich domains (excepting SRCR5), 42 peptides were matched to the β-sheets, and only two were matched to the corresponding α-helix of SRCR2 (residues 192–196) and SRCR9 (residues 954–960). No peptide appeared to interact with the transmembrane or intracellular domains, indicating that the interactions are limited to regions of the protein known to be extracellular. Supporting these observations, an anti-CD163 mAb inhibited binding of the CWDDGWSFC phage, which displays a peptide that matches several different regions of the extracellular domain of CD163, to TWEAK.

Other unexpected aspects of our results merit further discussion. The CD163 macrophage-restricted Ag is an established scavenger receptor of circulating Hp-Hb complexes (29, 36), but not of Hp or Hb separately (29). The binding region to CD163 is likely to be represented by structural components created by both Hp and Hb molecules or by an unfolding of a neo-epitope upon complex formation. The residues in the complexes containing the CD163-recognition site have not yet been characterized. However, the Hp β-chain (the major protein of the complex) harbors the receptor-recognition site exposed during complex formation because of its location in Hp (45, 46). Comparison of protein sequences of TWEAK with Hp and Hb reveals similarity in a region that comprised >30% of the secreted TWEAK. In addition, the hypervariable region of a Fab Ab that specifically recognizes the Hp-Hb complex revealed >40% homology with two TWEAK-binding peptides isolated from our panning, supporting the hypothesis that TWEAK may also mimic a neo-epitope generated in the Hp-Hb complex (37).

Madsen et al. (39) have recently shown that the anti-CD163 Ab clone Ki-M8 blocks the binding of the ligand complexes to SRCR domain 3 while clone GHI/61 binds to SRCR domain 7, not affecting the specific ligand-binding site. In this study we have shown that TWEAK binds in vitro to purified CD163 in a dose-dependent manner and that this binding is inhibited by preincubation of CD163 with mAbs raised against CD163. Moreover, TWEAK binding to Fn14/TweakR or CD163 can be displaced by the addition of the opposite soluble receptor, indicating a competition between both receptors. These results lend further support to our hypothesis of a TWEAK-CD163 protein interaction.

Because CD163 is expressed exclusively on monocytes and macrophages, we investigated the putative interactions of TWEAK and CD163 in this cell lineage. In agreement with previous reports (9), the established TWEAK receptor is not present at detectable levels on the surface of freshly isolated CD14-positive monocytes from healthy human donors. Nevertheless, such cells are capable of binding TWEAK as shown here. Thus, CD14-positive/CD163-positive cells not expressing Fn14/TweakR still bind TWEAK.

These results suggest that other receptor(s) may mediate the interaction. We show that preincubation with TWEAK inhibited recognition by a specific Ab of CD163 on the monocyte surface.

Therefore, we used cross-linking of CD163 by a specific Ab to activate tyrosine kinases in human monocytes. Given that the CD14-positive human monocytes in our system were nonproliferating (40), we demonstrated that incubation with an anti-CD163 Ab induced a metabolic activation relative to controls. Coincubation with TWEAK abrogated this activation. In contrast, monocytes incubated with TWEAK alone showed no change in the same conditions. Thus, TWEAK can inhibit the activating effect of the anti-CD163 mAb through biochemical competition for the binding site, steric hindrance, or both.

Also, we have shown that human monocytes can sequester soluble TWEAK from the culture medium. Consistently, monocytes are not able to bind TWEAK in the presence of either an anti-CD163 mAb or a peptide motif mimicking CD163. In light of these findings, TWEAK and CD163 seem to be functionally linked. In addition, TWEAK mediates monocyte death induced by autologous T cells in patients with certain autoimmune diseases (10) and previous reports have shown that TWEAK is produced by monocytes stimulated by IFN-γ, an anti-inflammatory cytokine that also negatively regulates CD163 (26), again suggesting that both proteins function in interacting pathways.

Finally, by using CHO cells transiently expressing the scavenger receptor CD163, we demonstrated that rHuTWEAK specifically binds to this receptor in a similar manner as to its natural receptor (Fn14/TweakR); such binding is reverted by unlabeled rHuTWEAK or the Hp-Hb complex. Moreover, the CD163 receptor also mediates rHuTWEAK degradation (data not shown), an observation consistent with a scavenger function for this ligand.

In summary, our results demonstrate that a protein-protein interaction occurs between TWEAK and CD163 at the cell surfaces of human CD14-positive monocytes. Several lines of evidence are presented in support of this hypothesis. First, phage clones displaying peptides that bind to TWEAK mimic the several SRCR domains in CD163. Furthermore, we also observed that the TWEAK protein structure mimics the Hp-Hb complex, an established CD163 ligand. TWEAK and CD163 also interact at the surface of Fn14/TweakR-negative human monocytes, and TWEAK competes for CD163 binding to these cells. Recently, it was reported that TWEAK promotes tumor cell invasion rather than apoptosis (47), an event that correlates with the overexpression of Fn14/TweakR. According to our findings, it could be proposed that CD163 is a scavenger receptor for TWEAK, thus preventing TWEAK from exerting its biological function(s) by sequestering it from the tumor environment. In accordance with the competition between TWEAK and Hp-Hb shown in the binding experiment, one might also propose that TWEAK could act as an antagonist for CD163. Future research efforts regarding the functional effects of TWEAK-CD163 interaction need to be performed to validate one of these hypotheses. A detailed understanding of this possible functional link between TWEAK and CD163 may have impact on conditions such as inflammation and cancer (10, 47, 48).

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Disclosures

The authors have no financial conflict of interest.

References

1. Locksley, R. M., N. Killeen, and M. J. Lenardo. 2001. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* 104: 487–501.
2. Ware, C. F. 2002. Tumor necrosis factors. In *Encyclopedia of Cancer*, 2nd Ed., J. R. Bertino, ed. Academic Press, San Diego, pp. 475–489.
3. Bodmer, J. L., P. Schneider, and J. Tschopp. 2002. The molecular architecture of the TNF superfamily. *Trends Biochem. Sci.* 27: 19–26.
4. Chicheportiche, Y., P. R. Bourdon, H. Xu, Y. M. Hsu, H. Scott, C. Hession, I. Garcia, and J. L. Browning. 1997. TWEAK, a new secreted ligand in the tumor necrosis factor family that weakly induces apoptosis. *J. Biol. Chem.* 272: 32401–32410.
5. Lynch, C. N., Y. C. Wang, J. K. Lund, Y. W. Chen, J. A. Leal, and S. R. Wiley. 1999. TWEAK induces angiogenesis and proliferation of endothelial cells. *J. Biol. Chem.* 274: 8455–8459.
6. Jakubowski, A., B. Browning, M. Lukashev, I. Sizing, J. S. Thompson, C. D. Benjamin, Y. M. Hsu, C. Ambrose, T. S. Zheng, and L. C. Burkly. 2002. Dual role for TWEAK in angiogenic regulation. *J. Cell Sci.* 115: 267–274.
7. Harada, N., M. Nakayama, H. Nakano, Y. Fukuchi, H. Yagita, and K. Okumura. 2002. Pro-inflammatory effect of TWEAK/Fn14 interaction on human umbilical vein endothelial cells. *Biochem. Biophys. Res. Commun.* 299: 488–493.
8. Chicheportiche, Y., R. Chicheportiche, I. Sizing, J. Thompson, C. B. Benjamin, C. Ambrose, and J. M. Dayer. 2002. Proinflammatory activity of TWEAK on human dermal fibroblasts and synoviocytes: blocking and enhancing effects of anti-TWEAK monoclonal antibodies. *Arthritis Res.* 4: 126–133.
9. Nakayama, M., N. Kayagaki, N. Yamaguchi, K. Okumura, and H. Yagita. 2000. Involvement of TWEAK in interferon γ-stimulated monocyte cytotoxicity. *J. Exp. Med.* 192: 1373–1379.
10. Kaplan, M. J., E. E. Lewis, E. A. Shelden, E. Somers, R. Pavlic, W. J. McCune, and B. C. Richardson. 2002. The apoptotic ligands TRAIL, TWEAK, and Fas ligand mediate monocyte death induced by autologous lupus T cells. *J. Immunol.* 169: 6020–6029.
11. Desplat-Jego, S., S. Varriale, R. Creidy, R. Terra, D. Bernard, M. Khestchatisky, S. Izui, Y. Chicheportiche, and J. Boucraut. 2002. TWEAK is expressed by glial cells, induces astrocyte proliferation and increases EAE severity. *J. Neuroimmunol.* 133: 116–123.
12. Nakayama, M., K. Ishidoh, N. Kayagaki, Y. Kojima, N. Yamaguchi, H. Nakano, E. Kominami, K. Okumura, and H. Yagita. 2002. Multiple pathways of TWEAK-induced cell death. *J. Immunol.* 168: 734–743.
13. Schneider, P., R. Schwenzer, E. Haas, F. Muhlenbeck, G. Schubert, P. Scheurich, J. Tschopp, and H. Wajant. 1999. TWEAK can induce cell death via endogenous TNF and TNF receptor 1. *Eur. J. Immunol.* 29: 1785–1792.
14. Kaptein, A., M. Jansen, G. Dilaver, J. Kitson, L. Dash, E. Wang, M. J. Owen, J. L. Bodmer, J. Tschopp, and S. N. Farrow. 2000. Studies on the interaction between TWEAK and the death receptor WSL-1/TRAMP (DR3). *FEBS Lett.* 485: 135–141.
15. Marsters, S. A., J. P. Sheridan, R. M. Pitti, J. Brush, A. Goddard, and A. Ashkenazi. 1998. Identification of a ligand for the death-domain-containing receptor Apo3. *Curr. Biol.* 8: 525–528.
16. Wiley, S. R., L. Cassiano, T. Lofton, T. Davis-Smith, J. A. Winkles, V. Lindner, H. Liu, T. O. Daniel, C. A. Smith, and W. C. Fanslow. 2001. A novel TNF receptor family member binds TWEAK and is implicated in angiogenesis. *Immunity* 15: 837–846.
17. Nakayama, M., K. Ishidoh, Y. Kojima, N. Harada, E. Kominami, K. Okumura, and H. Yagita. 2003. Fibroblast growth factor-inducible 14 mediates multiple pathways of TWEAK-induced cell death. *J. Immunol.* 170: 341–348.
18. Polek, T. C., M. Talpaz, B. G. Darnay, and T. Spivak-Kroizman. 2003. TWEAK mediates signal transduction and differentiation of RAW264.7 cells in the absence of Fn14/TweakR: evidence for a second TWEAK receptor. *J. Biol. Chem.* 278: 32317–32323.
19. Pasqualini, R., E. Koivunen, and E. Ruoslahti. 1995. A peptide isolated from phage display libraries is a structural and functional mimic of an RGD-binding site on integrins. *J. Cell Biol.* 130: 1189–1195.
20. Mintz, P. J., J. Kim, K. A. Do, X. Wang, R. G. Zinner, M. Cristofanilli, M. A. Arap, W. K. Hong, P. Troncoso, C. J. Logothetis, R. Pasqualini, and W. Arap. 2003. Fingerprinting the circulating repertoire of antibodies from cancer patients. *Nat. Biotechnol.* 21: 57–63.
21. Cardó-Vila, M., W. Arap, and R. Pasqualini. 2003. $\alpha_v\beta_5$ integrin-dependent programmed cell death triggered by a peptide mimic of annexin V. *Mol. Cell* 11: 1151–1162.
22. Pasqualini, R., E. Koivunen, R. Kain, J. Lahdenranta, M. Sakamoto, A. Stryhn, R. A. Ashmun, L. H. Shapiro, W. Arap, and E. Ruoslahti. 2000. Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis. *Cancer Res.* 60: 722–727.
23. Giordano, R. J., M. Cardó-Vila, J. Lahdenranta, R. Pasqualini, and W. Arap. 2001. Biopanning and rapid analysis of selective interactive ligands. *Nat. Med.* 7: 1249–1253.
24. Hogger, P., J. Dreier, A. Droste, F. Buck, and C. Sorg. 1998. Identification of the integral membrane protein RM3/1 on human monocytes as a glucocorticoid-inducible member of the scavenger receptor cysteine-rich family (CD163). *J. Immunol.* 161: 1883–1890.
25. Backe, E., R. Schwarting, J. Gerdes, M. Ernst, and H. Stein. 1991. Ber-MAC3: new monoclonal antibody that defines human monocyte/macrophage differentiation antigen. *J. Clin. Pathol.* 44: 936–945.
26. Buechler, C., M. Ritter, E. Orso, T. Langmann, J. Klucken, and G. Schmitz. 2000. Regulation of scavenger receptor CD163 expression in human monocytes and macrophages by pro- and anti-inflammatory stimuli. *J. Leukocyte Biol.* 67: 97–103.

27. Pulford, K., K. Micklem, S. McCarthy, J. Cordell, M. Jones, and D. Y. Mason. 1992. A monocyte/macrophage antigen recognized by the four antibodies GHI/61, Ber-MAC3, Ki-M8 and SM4. *Immunology* 75: 588–595.
28. Van den Heuvel, M. M., C. P. Tensen, J. H. Van As, T. K. Van den Berg, D. M. Fluitsma, C. D. Dijkstra, E. A. Dopp, A. Droste, F. A. Van Gaalen, C. Sorg, et al. 1999. Regulation of CD163 on human macrophages: cross-linking of CD163 induces signaling and activation. *J. Leukocyte Biol.* 66: 858–866.
29. Kristiansen, M., J. H. Graversen, C. Jacobsen, O. Sonne, H. J. Hoffman, S. K. Law, and S. K. Moestrup. 2001. Identification of the haemoglobin scavenger receptor. *Nature* 409: 198–201.
30. Komohara, Y., J. Hirahara, T. Horikawa, K. Kawamura, E. Kiyota, N. Sakashita, N. Araki, and M. Takeya. 2006. AM-3K, an anti-macrophage antibody, recognizes CD163, a molecule associated with an anti-inflammatory macrophage phenotype. *J. Histochem. Cytochem.* 54: 763–771.
31. Barbas, C. F., III, D. R. Burton, J. K. Scott, and G. J. Silverman. 2000. *Phage Display: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
32. Arap, W., M. G. Kolonin, M. Trepel, J. Lahdenranta, M. Cardó-Vila, R. J. Giordano, P. J. Mintz, P. U. Ardelt, V. J. Yao, C. I. Vidal, et al. 2002. Steps toward mapping the human vasculature by phage display. *Nat. Med.* 8: 121–127.
33. Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment though sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673–4680.
34. Virella, G., J. F. Muñoz, G. M. P. Galbraith, C. Gissinger, C. Chassereau, and M. F. Lopes-Virella. 1995. Activation of human monocyte-derived macrophages by immune complexes containing low-density lipoprotein. *Clin. Immunol. Immunopathol.* 75: 179–189.
35. Kuniyasu, A., N. Ohgami, S. Hayashi, A. Miyazaki, S. Horiuchi, and H. Nakayama. 2003. CD36-mediated endocytic uptake of advanced glycation end products (AGE) in mouse 3T3-L1 and human subcutaneous adipocytes. *FEBS Lett.* 537: 85–90.
36. Graversen, J. H., M. Madsen, and S. K. Moestrup. 2002. CD163: a signal receptor scavenging haptoglobin-hemoglobin complexes from plasma. *Int. J. Biochem. Cell Biol.* 34: 309–314.
37. Yoshioka, N., and M. Z. Atassi. 1986. Hemoglobin binding with haptoglobin: localization of the haptoglobin-binding sites on the β-chain of human haemoglobin by synthetic overlapping peptides encompassing the entire chain. *Biochem. J.* 234: 453–456.
38. Horn, I. R., M. J. Nielsen, M. Madsen, C. Jacobsen, J. H. Graversen, S. K. Moestrup, and C. Jacobsen. 2003. Generation of a haptoglobin-hemoglobin complex-specific Fab antibody blocking the binding of the complex to CD163. *Eur. J. Haematol.* 71: 289–293.
39. Madsen, M., H. J. Moller, M. J. Nielsen, C. Jacobsen, J. H. Graversen, T. van den Berg, and S. K. Moestrup. 2004. Molecular characterization of the haptoglobin.hemoglobin receptor CD163: ligand binding properties of the scavenger receptor cysteine-rich domain region. *J. Biol. Chem.* 279: 51561–51567.
40. Lund, P. K., E. Namork, S. H. Brorson, A. B. Westvik, G. B. Joo, R. Ovstebo, and P. Kierulf. 2002. The fate of monocytes during 24 h of culture as revealed by flow cytometry and electron microscopy. *J. Immunol. Methods* 270: 63–76.
41. Takase, S., G. Schmid-Schonbein, and J. Bergan. 1999. Leukocyte activation in patients with venous insufficiency. *J. Vasc. Surg.* 30: 148–156.
42. Idriss, H. T., and J. H. Naismith. 2000. TNF α and the TNF receptor superfamily: structure-function relationship(s). *Microsc. Res. Tech.* 50: 184–195.
43. Koths, K., E. Taylor, R. Halenbeck, C. Casipit, and A. Wang. 1993. Cloning and characterization of a human Mac-2-binding protein, a new member of the superfamily defined by the macrophage scavenger receptor cysteine-rich domain. *J. Biol. Chem.* 19: 14245–14249.
44. Hohenester, E., T. Sasaki, and R. Timpl. 1999. Crystal structure of a scavenger receptor cysteine-rich domain sheds light on an ancient superfamily. *Nat. Struct. Biol.* 6: 228–232.
45. Javid, J., and P. K. Pettis. 1975. The modification of hemoglobin-antihemoglobin reaction by haptoglobin. *J. Lab. Clin. Med.* 86: 777–784.
46. Wejman, J. C., D. Hovsepian, J. S. Wall, J. F. Hainfeld, and J. Greer. 1984. Structure and assembly of haptoglobin polymers by electron microscopy. *J. Mol. Biol.* 174: 343–368.
47. Tran, N. L., W. S. McDonough, B. A. Savitch, S. P. Fortin, J. A. Winkles, M. Symons, M. Nakada, H. E. Cunliffe, G. Hostetter, D. B. Hoelzinger, et al. 2006. Increased fibroblast growth factor-inducible 14 expression levels promote glioma cell invasion via Rac1 and nuclear factor-κB and correlate with poor patient outcome. *Cancer Res.* 66: 9535–9542.
48. Perper, S. J., B. Browning, L. C. Burkly, S. Weng, C. Gao, K. Giza, L. Su, L. Tarilonte, T. Crowell, L. Rajman, et al. 2006. TWEAK is a novel arthritogenic mediator. *J. Immunol.* 177: 2610–2620.