

The Vitronectin Receptor and its Associated CD47 Molecule Mediates Proinflammatory Cytokine Synthesis in Human Monocytes by Interaction with Soluble CD23

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Abstract. The vitronectin receptor, $\alpha_v\beta_3$ integrin, plays an important role in tumor cell invasion, angiogenesis, and phagocytosis of apoptotic cells. CD47, a member of the multispan transmembrane receptor family, physically and functionally associates with vitronectin receptor (VnR). Although vitronectin (Vn) is not a ligand of CD47, anti-CD47 and β_3 mAbs suppress Vn, but not fibronectin (Fn) binding and function. Here, we show that anti-CD47, anti- β_3 mAb and Vn, but not Fn, inhibit sCD23-mediated proinflammatory function (TNF- α , IL-12, and IFN- γ release). Surprisingly, anti-CD47 and β_3 mAbs do not block sCD23 binding to $\alpha_v^+\beta_3^+$ T cell lines, whereas Vn and an α_v mAb (clone

AMF7) do inhibit sCD23 binding, suggesting the VnR complex may be a functional receptor for sCD23. sCD23 directly binds $\alpha_v^+\beta_3^+/CD47^-$ cell lines, but co-expression of CD47 increases binding. Moreover, sCD23 binds purified α_v protein and a single human α_v chain CHO transfectant. We conclude that the VnR and its associated CD47 molecule may function as a novel receptor for sCD23 to mediate its proinflammatory activity and, as such, may be involved in the inflammatory process of the immune response.

Key words: CD47 • CD23 • vitronectin receptor • TNF- α • IFN- γ

THE vitronectin receptor ($\alpha_v\beta_3$) is an ubiquitous receptor that interacts with several ligands, such as vitronectin (Vn)¹, fibronectin (Fn), osteopontin, and metalloproteinase MMP-2 (for review see Felding-Habermann and Cheresch, 1993; Brooks et al., 1996). As a consequence, this integrin plays a role in diverse biologic processes such as cell migration, tumor invasion, bone resorption, angiogenesis, and immune responsiveness (Gladson and Cheresch, 1994).

During the process of inflammation, circulating human monocytes are able to leave the blood by attaching to, and migrating through, endothelial and subendothelial matrices to the site of injury. The vitronectin receptor (VnR)

$\alpha_v\beta_3$, expressed on endothelial cells, is involved in the transendothelial migration process (Brown and Lindberg, 1996) together with PECAM (CD31) (Buckley et al., 1996) and CD47 (Cooper et al., 1995). When recruited at the inflammatory or infected sites, phagocytes undergo a number of physiological changes including increases in adhesiveness, production of reactive oxygen metabolites, and augmentation in phagocytosis. Extracellular matrix proteins containing RGD sequence peptides have been shown to mediate some of these functions (Hynes, 1992), especially activation of phagocytic burst (Zhou and Brown, 1993), and enhancement of ingestion of opsonized particles by monocytes (Gresham et al., 1989).

The CD47 Ag is a widely expressed 50-kD multispan transmembrane protein component of the $\alpha_v\beta_3$ and leukocyte response integrin signaling complex, since its expression was shown to enhance Vn, but not Fn, binding and function to a variety of cells (Brown et al., 1990; Rosales et al., 1992; Lindberg et al., 1993). It was reported that CD47 does not directly bind Vn, and CD47⁻ cell lines, expressing $\alpha_v\beta_3$, failed to bind Vn-coated beads (Lindberg et al., 1996b). Moreover, CD47 deficient mice rapidly died of *Escherichia coli* peritonitis, a phenomenon directly cor-

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1. *Abbreviations used in this paper:* B, biotinylated; Fn, fibronectin; sCD23, soluble CD23; TSP, thrombospondin; Vn, vitronectin; VnR, vitronectin receptor.

related with a reduction in leukocyte activation in response to β_3 , but not β_2 , integrin ligation (Lindberg et al., 1996a). The $\alpha_v\beta_3$ /CD47 trimolecular complex also participates in the resolution of inflammation by mediating phagocytosis of aging leukocytes undergoing apoptosis before they disgorge their potentially harmful contents (Savill et al., 1990). This process is potentiated by the synthesis of proinflammatory cytokine such as GM-CSF, TNF- α , IL-1, and IFN- γ (Ren and Savill, 1995).

CD23 has been purported to play a role in inflammation based upon its *in vitro* proinflammatory activity (Armant et al., 1994; Leconet-Henchoz et al., 1995; Bonnefoy et al., 1996; Sarfati, 1997) and the observation that soluble CD23 (sCD23) levels increased in various chronic inflammatory disorders, including rheumatoid arthritis and systemic lupus erythematosus (Ikizawa et al., 1993; Bertero et al., 1994). sCD23 (Armant et al., 1994; Leconet-Henchoz et al., 1995) and CD23 ligation (Bonnefoy et al., 1996) can trigger monokine release by human monocytes. Our studies have demonstrated that sCD23-induced TNF- α secretion costimulates IFN- γ production by IL-2-activated T cells cocultured with syngeneic monocytes in the absence of T cell receptor ligation (Armant et al., 1995).

Here, we report a novel function for VnR and its associated CD47 molecule on monocytes, by demonstrating that this trimolecular complex mediates proinflammatory cytokine synthesis via interaction with CD23. This may contribute to the perpetuation of the inflammatory process in chronic disorders such as rheumatoid arthritis. In this disease, CD23, TNF- α , and VnR expression are found to be locally elevated in the inflamed synovium (Ashton et al., 1993; Feldman et al., 1996).

Materials and Methods

Cell Lines and Reagents

Human recombinant IL-2, kindly provided by Dr. D. Bron (Institut Bordet, Brussels, Belgium), was used at 20 U/ml; IL-15 was obtained from Immunex and used at 200 ng/ml. Endotoxin-free (<15 pg/ml, as determined by the chromogenic Limulus amoebocyte lysate, QCL-1000, BioWhittaker Inc.) affinity-purified sCD23 was prepared in our laboratory from CSN of CHO cell line transfected with human cDNA encoding for aa 148–321 of the CD23 molecule. The concentration of 25 ng/ml sCD23 used throughout this study was selected on the basis of previously reported dose-response curves (Armant et al., 1994). Jurkat T ($\alpha_v^+\beta_3^+$), THP-1 ($\alpha_v^-\beta_3^-$) monocytic, Raji ($\alpha_v^+\beta_3^-$), and Bowes melanoma ($\alpha_v^+\beta_3^+$) cell lines were obtained from the American Type Culture Collection (ATCC). K562 and K562 transfected with the cDNA encoding the full-length CR2 (K562-CR2) were a generous gift from Drs. A. Masumoto and D. Fearon (Johns Hopkins University, Baltimore, MD). CD47 deficient Jurkat T cell line and OV10 ovarian carcinoma cell line were generated in Drs. E. Brown and F. Lindberg's laboratory (Washington University, St. Louis, MO). cDNA encoding for CD51 (α_v chain) was a generous gift from Dr. E. Ruoslahti (Burnham Institute, La Jolla, CA). 10G2 mAb (IgM class) was produced in our laboratory following immunization of mice with Jurkat T cells. Hybridomas producing anti-CD47 (clone B6H12) and anti- β_3 (CD61) mAbs (clone AP3) were purchased at the ATCC. Anti- $\alpha_v\beta_3$ (CD51/CD61, clone LM609), and anti- α_v (CD51, clone LM142) were kindly provided by Dr. Cheresch (Scripps Research Institute, La Jolla, CA). Anti- α_v (CD51, clone AMF7) and anti-CD47 (clone BRIC126, CKm1) were purchased from Immunotech, Serotec Ltd., and Accurate Chemical and Scientific Corp., respectively. RGDS and RGEs peptides, Vn, Fn, and thrombospondin (TSP) were obtained from GIBCO BRL.

Expression Cloning of 10G2 Antigen (CD47)

COS cells were transfected using the DEAE dextran method, with an ex-

pression library derived from Jurkat cells (4×10^5 clones). Cells expressing the molecule recognized by the 10G2 mAb were immunoselected by indirect panning using 10G2 and anti-mouse IgM-coated plates. Specifically bound cells were lysed, plasmid DNA was isolated, amplified, and retransfected into COS cells. Following an additional round of immunoselection and plasmid purification, pools of 150 clones each were transfected into COS cells, which were subsequently screened for positivity by incubation with radiolabeled 10G2 mAb. Two rounds of screening resulted in the isolation of a single 10G2-reactive clone.

Establishment of Stable CHO Transfectants

CHO cells were grown at 50% confluence in 150-cm² culture flasks in 5% heat decomplemented FCS containing DME (GIBCO BRL) supplemented with 2 mM glutamine, 100 IU penicillin, and 100 μ g/ml streptomycin. Cells were harvested using versene solution (GIBCO BRL) and washed twice with pure DME. Cells were then resuspended at 11×10^6 cells/ml, and 10^7 cells were incubated in a 0.4-cm electrotransfection cuvette (BioRad Laboratories) for 10 min with 20 μ g of pBJ α_v (plasmid containing cDNA encoding the full-length α_v molecule, CD51), in order to obtain CHO cells expressing the α_v molecules. Cells were then pulsed at 220 V and 960 μ F using a Gene Pulser (BioRad Laboratories). After another 10 min of incubation at room temperature, electroporated cells were grown for 24 h in nonselective culture medium which was then replaced by complete DME containing 500 μ g/ml active G418 (GIBCO BRL). After 14 d of culture, the pool of survivors was analyzed by flow cytometry, and CHO cell line expressing α_v was subsequently enriched by FACS[®] for the 5% highest stained cells using anti-CD51 mAb as primary antibody. After four rounds of sorting, we obtained stable cell lines, namely CHO-51, containing >99% of CD51⁺ cells.

Cell Separation and Culture Conditions

Monocytes. PBMC were isolated by density gradient centrifugation of heparinized blood from healthy volunteers using Lymphoprep (Nycomed). Monocytes were prepared by cold aggregation as described in Armant et al. (1995). Monocyte purity was shown to be >95% using phycoerythrin-conjugated anti-CD14 mAb and flow cytometry (Becton Dickinson and Co.). Cellular viability was >90% using trypan blue exclusion.

T Cells. Enriched T cell populations were obtained from the monocyte-depleted PBMC by rosetting with AET-SRBC and treatment with ammonium chloride. To obtain highly purified T cells, rosette forming cells were washed and incubated for 20 min at 37°C in Lympho-Kwik T (One Lambda). Cell purity was assessed by flow cytometry (FACScan[®]; Becton Dickinson and Co.) using phycoerythrin-conjugated anti-CD3 mAb (Becton Dickinson and Co.) and shown to be >98% in all cases.

Cultures were performed in complete serum-free HB101 medium (Irvine Scientific) supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 10 mM Hepes, 100 IU penicillin, and 100 μ g/ml streptomycin in the presence of polymyxin B 10 μ g/ml (Sigma Chemical Co.). When cultured alone, monocytes were incubated in 5 ml sterile Falcon tubes (Becton Dickinson and Co.) at 2×10^5 cells/ml for cytokine measurement. For coculture experiments, T cells (10^6 cells/ml) were incubated with monocytes (2×10^5 cells/ml) in 24-well Falcon plates.

Cytofluorometric Analysis

For sCD23 binding, cells or cell lines were washed in HBSS (Gibco Laboratories), resuspended in HB101 complete serum free medium containing biotinylated sCD23 (B-sCD23; 50 ng/ml) in the absence or presence of mAbs (CD47, CD51, CD51/CD61), soluble Vn or Fn (20 μ g/ml), and RGDS peptides (20 μ g/ml). After 4–6 h of incubation at 22°C, cells were washed with PBS containing 3% BSA, and further incubated with phycoerythrin-streptavidin (Becton Dickinson and Co.). Cell viability assessed by trypan blue exclusion was >85% before staining with fluorochrome. Indirect immunofluorescence staining of cells or cell lines with different mAbs was performed according to standard techniques (Armant et al., 1995).

Lymphokine Determinations

IFN- γ , TNF- α , IL-10, and IL-12 were measured exactly as described in Armant et al. (1994, 1995). IL-1 β , IL-8, and PGE₂ were measured by ELISA kits purchased from R & D Systems, Inc.

Table I. Cellular Distribution of 10G2 Antigen on Human Cell Lines

	Freshly isolated human leukocytes		Human cell lines		
	sCD23	10G2	sCD23	10G2	
	MFI	MFI	MFI	MFI	
T cells	+++	++++	T cell lines (Jurkat, CEM, HUT 78)	++	++++
B cells	++	++	B cell lines (RPMI 8226, Raji, WIL-2, Daudi)	++	++
Monocytes	+	+	Monocyte cell lines (U937)	++	++
			THP-1	-	-
Erythrocytes	-	-	Erythroleukemia cell lines (K562/K562-CR2)	+	+

Range of mean fluorescence intensity (MFI) are indicated as follows: (+) 1-5; (++) 10-15; (+++) 20-25; (++++) 25-35.

Immunoprecipitation and Western Blot Analysis

Cells were lysed in PBS containing 1% NP-40 (NP-40/PBS) supplemented with protease inhibitors. The lysate was purified on anti- β_3 affinity column, and the eluate was separated by SDS-PAGE (5%) under nonreducing conditions and transferred to PDVF membrane (Millipore Corp.). Nonspecific binding sites on the membrane were blocked with PBS containing 5% milk. The membrane was incubated with milk containing B-BSA, B-sCD23, B-CD51 mAbs (clone AMF7 and LM142), or B-CD61 mAb. After overnight incubation, membrane was treated with avidin-DH and biotinylated peroxidase complex (Vectastain, ABC kit, Vector Labs, Inc.) followed by ECL detection reagent (Nycomed Amersham).

Statistical Analysis

Paired *t* tests have been used to assess levels of significance (**P* < 0.05; ***P* < 0.01; ****P* < 0.001).

Results

10G2 mAb, Which Inhibits sCD23 Biological Activities, Recognizes CD47 Ag

sCD23 displays potent proinflammatory activity by directly triggering monokine release from purified monocytes in the absence of costimulatory signals, such as bacterial antigens (LPS or SAC) or T cell-dependent signal (sCD40L; Armant et al., 1994, 1995; Leconaet-Henchoz et al., 1995). Although CD21 (CR2) and CD11b (CR3) were previously described as novel CD23 counterreceptors (Aubry et al., 1992; Leconaet-Henchoz et al., 1995), we also detected binding of sCD23 to several T cell lines lacking CR2 or CR3 expression (Ishihara et al., 1995; Table I). In an effort to identify sCD23 binding component, we generated mAbs to Jurkat T cells. We identified one mAb, clone 10G2, which neutralized sCD23 biological activities (Fig. 1 A), and displayed similar cell reactivity as sCD23 (Table I). Specifically, 10G2 mAb, like sCD23, did not stain the CD11b⁺ (CR3) and CD11c⁺ (CR4) THP-1 cell line. It recognized weakly, but with similar intensity, K562 and K562-CR2 cell lines. 10G2 mAb also stained peripheral blood T, B cells, and monocytes (Table I). 10G2 mAb significantly suppressed sCD23 costimulation of IFN- γ production by IL-2-stimulated T cells cocultured with autologous monocytes (mean inhibition of 10 experiments, 66%, *P* < 0.03; Fig. 1 A).

The molecule recognized by 10G2 mAb was identified by immunoaffinity panning of COS cells transfected with a cDNA library prepared from Jurkat T cells. After several rounds of selection, a single clone was selected which was found by DNA sequencing to encode CD47 Ag (data not

shown). Results in Fig. 1 B demonstrate binding of 10G2 to COS cells transfected with CD47 cDNA clone.

The effects of several anti-CD47 mAbs on sCD23 biological activity were examined (Fig. 1 A). Besides the 10G2 mAb (IgM isotype), three other anti-CD47 mAbs of different Ig isotype subclasses, C1Km1 (IgG1), BRIC126 (Ig2b), and B6H12 (IgG1) inhibited IFN- γ production induced by sCD23 plus IL-2. These results support the notion that CD47 is part of the signaling complex triggered by sCD23.

However, it appears that 10G2 was preferentially binding to a particular epitope on CD47. As depicted in Fig. 1 C, THP-1 cells express CD47 Ag but are not recognized by

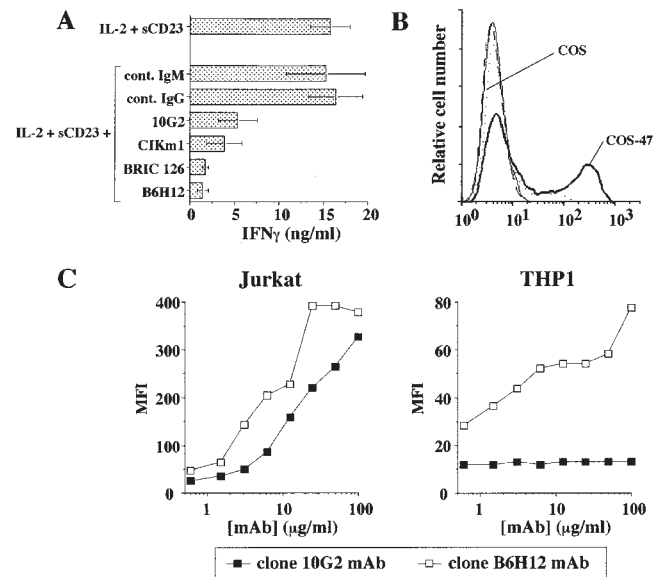


Figure 1. Clone 10G2 neutralizes sCD23 biological activities and recognizes CD47 Ag. (A) Inhibition of sCD23 costimulation of IFN- γ production in T cell/monocyte cocultures by anti-CD47 mAbs (clones 10G2, B6H12, C1Km1, and BRIC126). Mean \pm SD for 10G2 *P* < 0.03 (ten experiments) and for other mAbs *P* < 0.001 (five independent experiments). (B) 10G2 mAb staining of untransfected (COS, dotted line) or CD47-transfected COS cell line (COS-47, bold line). Control mAb staining of both cell lines: (COS, thin line; COS-47, dashed line). (C) Fluorescence staining of Jurkat T cell line (left) and THP-1 monocyte cell line (right) by increasing concentrations of anti-CD47 mAbs (clones 10G2 and B6H12). One representative experiment out of three.

10G2 mAb. Clone B6H12, a well-defined anti-CD47 mAb, but not 10G2, stained THP-1 cell line in a dose-dependent manner, while Jurkat T cells were recognized with similar intensity by both anti-CD47 mAbs. There was no cross-inhibition of Jurkat staining by the two clones (data not detailed). Our unpublished data also indicated that erythrocytes which expressed CD47 in the absence of integrins (Rosales et al., 1992) were stained by B6H12 but not 10G2.

Anti-CD47 mAbs Suppress CD23 Costimulation of IL-12 and IFN- γ Production Via Fc-independent Pathways

We investigated the mechanisms underlying the suppression of IFN- γ production by anti-CD47 mAbs. In addition to 10G2 mAb of the IgM isotype, the F(ab')₂ fragments of B6H12 or intact BRIC126 mAb suppressed, in a dose-dependent manner, the ability of IL-2 and sCD23 to augment the production of IFN- γ by T cells cocultured with monocytes, demonstrating that the inhibition of IFN- γ se-

cretion by anti-CD47 mAb was not Fc-mediated (Fig. 2 A). Interestingly, IFN- γ secretion by IL-2-stimulated T cells cocultured with monocytes and graded numbers of CD23-transfected CHO cells, was also abrogated by anti-CD47 mAbs (Fig. 2 B).

We previously reported in the presence of IL-2 or IL-15, low levels of CD40L, expressed by unstimulated T cells, were sufficient to engage CD40 on monocytes and trigger IL-12 release. This monocyte-derived IL-12 production synergized with IL-2 or IL-15 to augment IFN- γ production by T cells (Armant et al., 1996; Avice et al., 1998). The IFN- γ response could be further amplified by sCD23-induced TNF- α release (Armant et al., 1995). Although sCD23 did not trigger IL-12 production by purified monocytes (Armant et al., 1995), it costimulated IFN- γ and IL-12 release in this coculture system, and the secretion of both cytokines was strongly inhibited by anti-CD47 mAbs (Fig. 2, C and D).

Anti-CD47 mAb Suppresses sCD23-induced Monokine Release

Because sCD23 directly triggers TNF- α release by purified monocytes (Armant et al., 1995), and monocytes express CD47 (Table I), we examined the effect anti-CD47 mAb had on sCD23-induced monokine release by monocytes. Anti-CD47 mAb significantly suppressed the induction by sCD23 of TNF- α , IL-1 β , and PGE₂ without affecting IL-8 secretion (Fig. 3). The data (i.e., inhibition of TNF- α and IL-12 production) provide a mechanism by which anti-CD47 mAb can suppress sCD23 costimulation of IFN- γ production.

However, anti-CD47 mAb, in the absence of sCD23 or in the presence of LPS, did not modulate TNF- α production (Fig. 3 A). Our unpublished observations revealed that anti-CD47 mAb, used alone or in combination with sCD23, did not induce the production of monocyte deactivators (such as IL-10 or TGF- β), nor did it modulate SAC-induced TNF- α , IL-6, or IL-10 release. These results support the hypothesis that anti-CD47 mAbs interfere with the sCD23 signaling pathway without impairing general monocyte function.

sCD23 and Vitronectin Share the Same Receptor: VnR/CD47 Complex

Several studies indicate that CD47 is physically and functionally associated with the vitronectin receptor, $\alpha_v\beta_3$. Both anti-CD47 and anti- β_3 (CD61) mAbs can block binding of Vn, but not Fn, to $\alpha_v\beta_3$, even though Vn does not bind to CD47 (Brown et al., 1990; Lindberg et al., 1993). Therefore, we examined the biological effects of anti- β_3 mAb and the natural ligands of VnR (Vn and Fn) on sCD23 function. It is important to note that all cultures were performed in HB101 serum-free medium to eliminate FCS as a source of Vn and Fn. The results (Fig. 4) suggested that Vn and sCD23 might share the same receptor, namely VnR/CD47 complex. Anti- β_3 mAb suppressed sCD23 function, as defined by TNF- α secretion and IFN- γ production (Fig. 4 A, and data not shown). Furthermore, soluble Vn, but not Fn, suppressed sCD23-induced TNF- α release by purified monocytes (Fig. 4 B). sCD23 and Vn most likely bound to distinct epitopes on $\alpha_v\beta_3$, since an

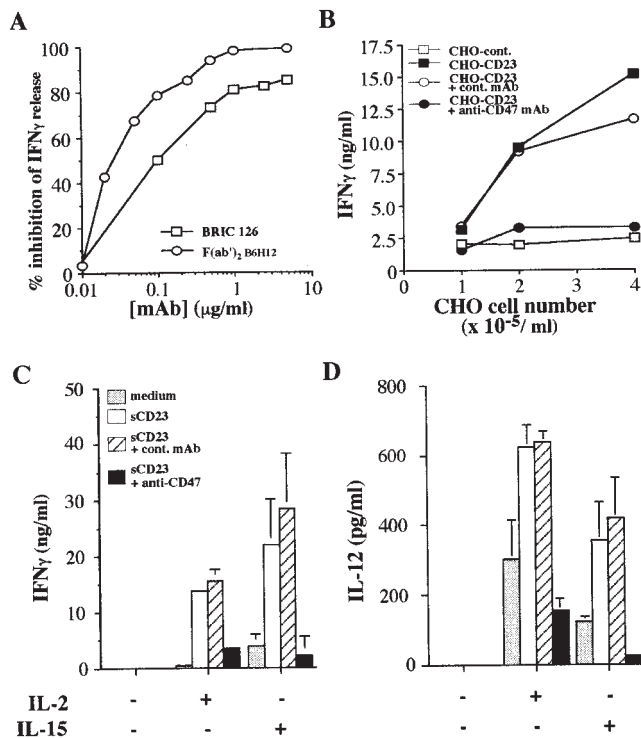


Figure 2. Anti-CD47 mAb inhibits CD23 costimulation of IFN- γ production. (A) Dose-dependent inhibition of IFN- γ production in IL-2 plus sCD23 stimulated coculture system by mAb clone BRIC126 and F(ab')₂ fragments of mAb clone B6H12. One representative experiment out of two. (B) Anti-CD47 mAb (clone B6H12) inhibition of IFN- γ secretion by T cells cocultured with autologous monocytes and increasing numbers of untransfected or CD23-transfected CHO cells. Similar data were obtained using clone 10G2 in three independent experiments. (C and D) Anti-CD47 mAbs (clone B6H12) mediated inhibition of IFN- γ and IL-12 secretion in cocultures of IL-2- or IL-15-stimulated T cells and autologous monocytes in the absence or presence of sCD23. Mean \pm SD of six experiments. Anti-CD47 mAb inhibition in IL-2- or IL-15-stimulated cocultures, for IFN- γ production $P < 0.001$, and $P < 0.01$ for IL-12 secretion.

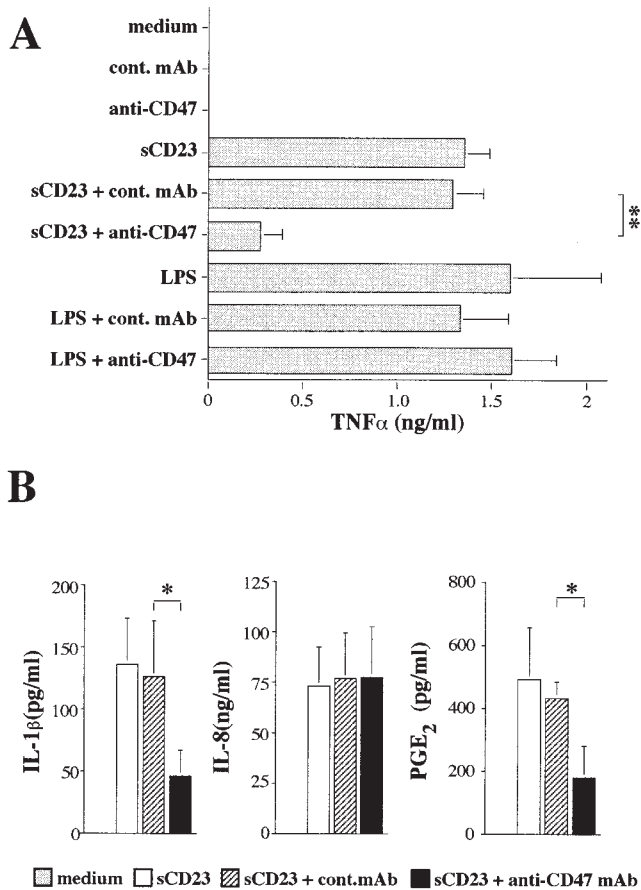


Figure 3. Anti-CD47 mAb inhibition of sCD23-induced monokine release by purified monocytes. Monocytes ($10^6/\text{ml}$) were cultured in the absence or presence of sCD23 or LPS with or without anti-CD47 mAb (clone 10G2). After overnight cultures, TNF- α (A), IL-1 β , IL-8, or PGE₂ (B) was measured in the culture supernatant. Mean \pm SD of six experiments (* $P < 0.05$, ** $P < 0.01$).

anti- $\alpha_v\beta_3$ mAb (clone LM609), which specifically inhibited Vn binding and function (Gao et al., 1996a), and RGDS peptide had no suppressive activity (Fig. 4 A, and data not shown).

We next investigated the ability of mAbs directed to the VnR complex, anti-CD47, CD61 (β_3) and $\alpha_v\beta_3$ (LM609) mAbs, and natural ligands of VnR, Vn, Fn, and RGDS peptides, to alter sCD23 binding to the Jurkat T cell line. Unexpectedly, anti-CD47 mAbs, alone or in combination (Fig. 5 b), anti-CD61 (Fig. 5 c), or anti- $\alpha_v\beta_3$ clone LM609 (not shown) did not inhibit sCD23 binding to Jurkat or monocytes (Hermann, P., unpublished observations). Note that sCD23 binding was specifically suppressed by anti-CD23 mAb (Fig. 5 a). The data suggested ligation of the CD47 or β_3 chain of the trimolecular complex by mAbs could indirectly inhibit sCD23 function by providing a negative signal to the target cells, or by modifying CD47 complex configuration without displacing the sCD23 molecule. We examined whether engagement of VnR/CD47 complex by its natural ligands would modify sCD23 binding. As shown in Fig. 5 d, Vn, but not Fn or RGDS peptide (not shown), significantly inhibited sCD23 binding, strongly

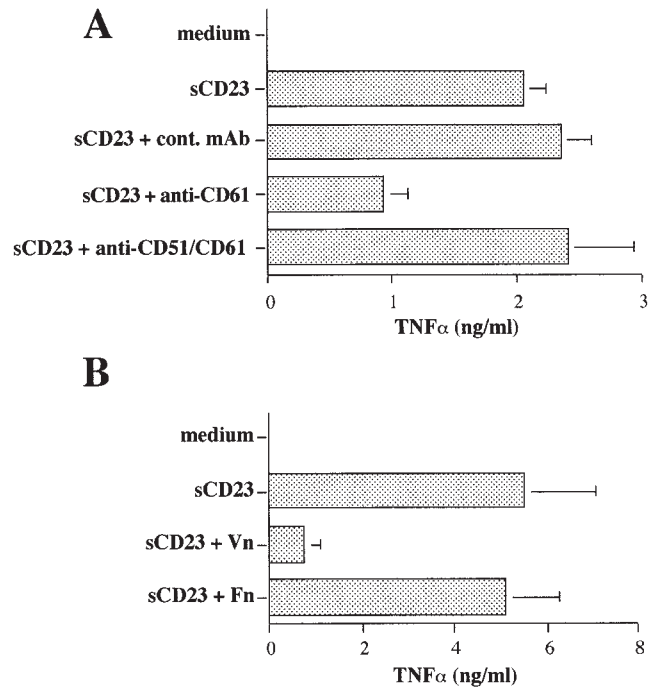


Figure 4. Suppression of sCD23-induced TNF- α release by anti- β_3 (CD61) mAb and Vn. sCD23-activated monocytes were cultured with anti- β_3 (CD61) clone AP3, anti- $\alpha_v\beta_3$ (CD51/CD61; clone LM609), or isotype-matched mAbs (A) and with soluble Vn or Fn (20 $\mu\text{g}/\text{ml}$, B). Mean \pm SD of four experiments ($P < 0.01$).

suggesting the VnR complex was involved in the secretion of proinflammatory cytokine via interaction with sCD23.

The possible role of the α_v chain (CD51) of VnR in sCD23 binding was explored. Three anti-CD51 mAbs were tested, and we found one anti-CD51 mAb (clone AMF7) inhibited the interaction between sCD23 and $\alpha_v^+\beta_3^+$ Jurkat T (Fig. 5 e), as well as $\alpha_v^+\beta_3^-$ Raji B cell lines (Fig. 5 f). Note that the Raji B cell line expresses the β_5 integrin (data not shown). We postulated that sCD23 was binding to α_v and not β_3 chain of the VnR, whereas β_3 and CD47 chains were likely to be involved in the signaling of the trimolecular complex in monocytes because anti-CD47 and anti- β_3 inhibited sCD23 function, but not binding.

sCD23 Interacts with $\alpha_v\beta_3$ and CD47 Coexpression Increases its Binding

Next, we selected a melanoma cell line, strongly expressing $\alpha_v\beta_3$ to purify this integrin by affinity chromatography using anti- β_3 (CD61) immobilized AFFi-gel. Western blot analysis (Fig. 6) shows sCD23 (lane 6) reacted with a single band of ~ 135 kD, displaying a similar migration pattern as molecular species recognized by a cocktail of anti-CD51 mAbs (lane 2). However, sCD23 did not appear to bind purified β_3 (CD61) chain which was identified by anti-CD61 mAb (lane 4), or purified recombinant CD47 (not shown).

To further support the hypothesis that sCD23 may directly interact with α_v chain of the trimolecular complex,

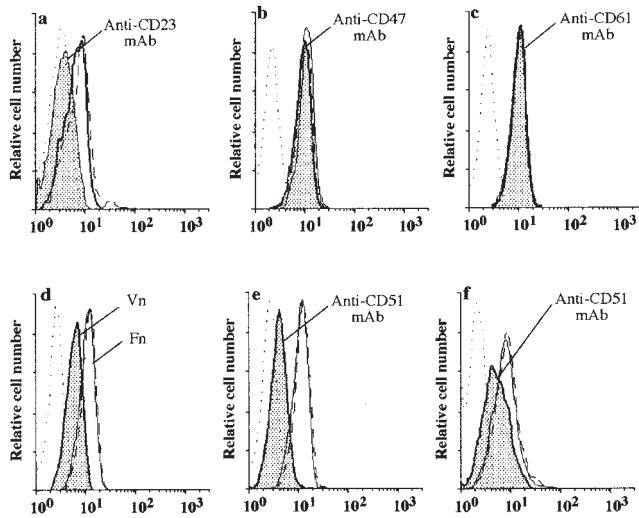


Figure 5. Effect of mAbs or natural ligands to VnR/CD47 complex on sCD23 binding to Jurkat and Raji cell lines. Jurkat $\alpha_v^+\beta_3^+$ (CD51⁺/CD61⁺) (a–e), or Raji $\alpha_v^+\beta_3^-$ (CD51⁺/CD61⁻) (f) cell lines were stained by B-BSA (dotted line) or B-sCD23 in the absence (solid line) or presence of the following inhibitors (plain histograms): anti-CD23 mAb (a); anti-CD47 mAbs (cocktail of four anti-CD47 mAbs; b); anti- β_3 (CD61) mAb (c); Vn (d); anti- α_v (CD51) mAb (e and f). B-sCD23 plus isotype-matched control mAb (a–c, e, and f) or Fn (d) were shown as dashed lines. One representative experiment out of four.

we prepared CHO transfectants singly expressing human α_v chain (CHO-CD51). The results in Fig. 7 demonstrated sCD23 strongly bound to CHO-CD51 compared to untransfected cell line. Although CHO-CD51 transfectant did not express human β_3 (CD61) chain, CHO cell lines were reported to express rodent β chain integrins (Lindberg et al., 1993) which likely associated with the human α_v chain underlying the successful stable expression of a single human integrin chain. Nevertheless, CHO cells also expressed hamster CD47 which might contribute to

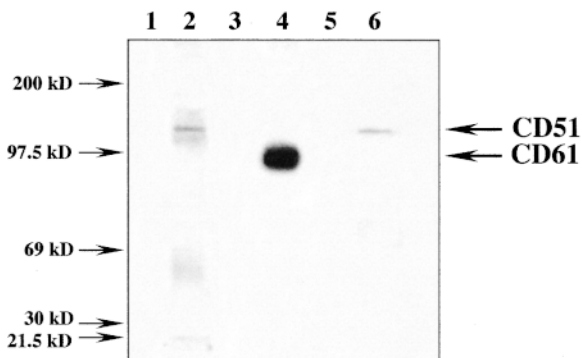


Figure 6. Western blot analysis of $\alpha_v^+\beta_3^+$ (CD51⁺/CD61⁺) melanoma cell line. Cell lysate was purified on anti- β_3 (CD61) mAb-affinity column. The eluate was separated by SDS-PAGE (5%) and transferred to membrane. Staining by anti- α_v (CD51) mAb (clone AMF7 and LM142; lane 2), anti- β_3 (CD61, clone AP3; lane 4), isotype-control matched mAbs (lanes 1 and 3), B-BSA (lane 5), and B-sCD23 (lane 6) is shown.

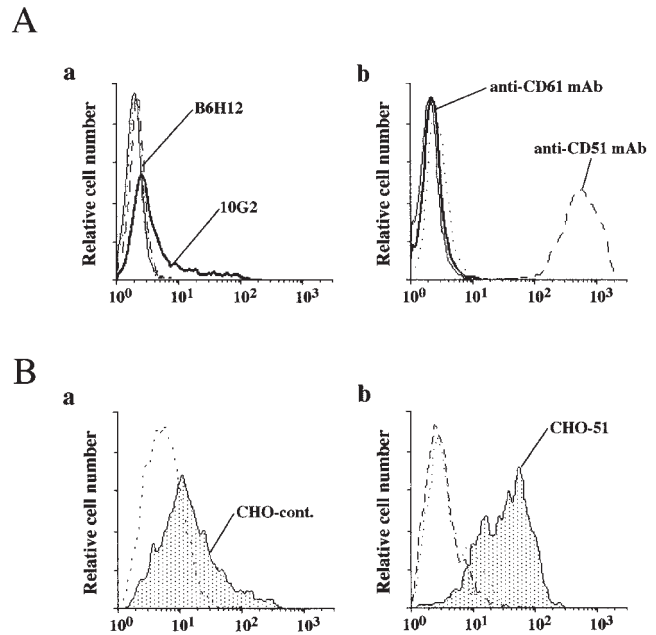


Figure 7. Binding of sCD23 to CHO-51 (CHO α_v) transfected cell line. (A) α_v (CD51)-transfected CHO cell line was stained with (a) anti-CD47 mAbs (clones 10G2, solid line and B6H12, dotted line) or isotype-matched control mAbs or with (b) anti- α_v (CD51) mAb (clone AMF7, dashed line) and anti- β_3 (CD61) mAb (clone AP3, solid line), or isotype-matched cont mAbs. (B) Untransfected (a), and α_v (CD51)-transfected CHO cell lines (b) were stained with B-sCD23 (plain histograms) or B-BSA (dotted line). One representative experiment out of three.

sCD23 binding to α_v /CD51 on live cells as reported for Vn binding to untransfected CHO cells (Lindberg et al., 1993). To directly assess whether CD47 expression was required for sCD23 interaction with α_v /CD51, we examined sCD23 binding to human CD47 deficient cell lines. As shown in Fig. 8, sCD23 bound to OV10 ovarian carcinoma VnR⁺/CD47⁻ cell line demonstrating that CD47 was dispensable for sCD23 binding. Coexpression of CD47 further increased its binding. A similar effect was seen on transfection of CD47⁻ Jurkat with CD47 (data not shown).

Given that sCD23 was not binding to CD47⁺/ α_v^- (THP-1 cell line; Fig. 8), but reacted with $\alpha_v^+\beta_3^-$ (Raji cell line; Fig. 5 f), we concluded that sCD23 ligated α_v /CD51. The VnR complex ($\alpha_v\beta_3$ /CD47 and/or $\alpha_v\beta_x$ /CD47) was used as a functional receptor for sCD23 to mediate its proinflammatory activity and, as such, may be involved in the inflammatory process of the immune response.

Discussion

Our results can be summarized in the schematic model presented in Fig. 9. We first postulate (Fig. 9 A) that sCD23 and Vn have distinct recognition sites on the $\alpha_v\beta_3$ /CD47 complex. sCD23 binds to α_v (CD51) and Vn to $\alpha_v\beta_3$ (CD51/CD61) conformational site. Binding of Vn may induce structural changes in the integrin complex leading to the masking of sCD23 binding sites, and vice versa. This hypothesis is based on observations that Vn, anti- α_v mAb

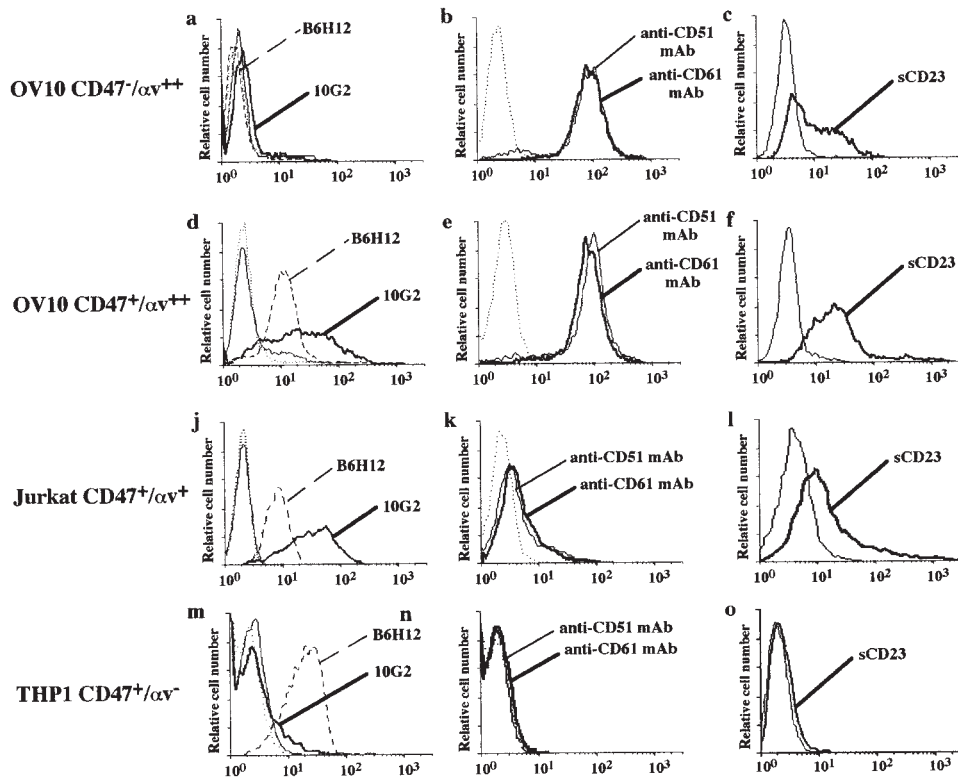


Figure 8. Binding of sCD23 to CD47⁺ and CD47⁻ cell lines. CD47⁺ and CD47⁻ cell lines were stained with two anti-CD47 mAbs (clone 10G2 and B6H12), anti- α_v (CD51) and anti- β_3 (CD61) mAbs, or B-sCD23 as described in Materials and Methods. OV10 carcinoma and Jurkat cell lines express different levels of α_v (CD51) chain which correlate with sCD23 binding.

(clone AMF7), but not clone LM609 (which specifically recognizes Vn binding site), nor RGDS peptide inhibited sCD23 binding and function (Figs. 4 and 5), and sCD23 directly bound to α_v chain (Figs. 6–8). However, CD47 was not a direct ligand for sCD23 while its coexpression improved sCD23 binding to α_v /CD51. sCD23 bound CD47 deficient cell lines, but failed to bind CD47 in the absence of α_v /CD51 integrin (THP-1 cell line, erythrocytes and recombinant CD47 protein; Fig. 8, and data not shown).

Secondly, (Fig. 9, B and C) CD47 or CD61 engagement by mAbs prevents Vn binding (Lindberg et al., 1993) without displacing sCD23 molecule (Fig. 5) while these mAbs inhibit both sCD23 (Figs. 2–4) and Vn function perhaps by modifying β_3 conformation or signaling pathway. Lindberg et al. (1993) indicated that anti-CD47 and anti- β_3 (CD61) mAbs (directed against an epitope of β_3 located outside the Vn binding site) inhibited Vn-opsonized particle binding and function. They also proposed the CD47 molecule participated in the appropriate folding of $\alpha_v\beta_3$, and modulated the affinity of $\alpha_v\beta_3$ for Vn (Brown et al., 1990; Lindberg et al., 1993). Vn-coated particles failed to bind to a CD47 deficient cell line (Lindberg et al., 1996b) or to cells isolated from CD47 deficient mice (Lindberg et al., 1996a), while these cells expressed $\alpha_v\beta_3$, demonstrating that CD47 is dispensable for VnR expression, but required for Vn binding and function. Using truncated forms of CD47, they also reported the interaction between the extracellular domain of CD47 (IgV) and α_v integrins was sufficient for Vn binding (Lindberg et al., 1996b).

It has been reported that adhesion of integrins to their counterreceptors is a dynamic phenomenon regulated by intracellular signal transduction pathways (Diamond and

Springer, 1994). Structural changes in the extracellular domains of integrins following mAb ligation may modify ligand adhesiveness in inducing or inhibiting ligand binding (inside-out signaling), or may directly provide a negative signal to the cell (outside-in signaling), as proposed here for anti-CD47 and anti-CD61 mAb-mediated inhibition of sCD23 function.

In agreement with this hypothesis, it was reported that antibodies recognizing CD81, another member of multi-span transmembrane receptors family, inhibited Fc ϵ RI-mediated mast cell degranulation without affecting IgE binding, receptor-mediated Ca²⁺ release, or tyrosine phosphorylation (Fleming et al., 1997).

Therefore, we propose a novel function for VnR/CD47 complex in the regulation of inflammatory response. In the absence of pathogen, ligation of VnR by sCD23 mediates monokine release such as TNF- α which enhances the inflammatory process by triggering the cascade of proinflammatory cytokine secretion (IL-1, IL-6, GM-CSF . . . ; Feldman et al., 1996), and facilitates the elimination of apoptotic cells (Ren and Savill, 1995). Both effects are negatively regulated by the presence of Vn (Fig. 4 B and Savill et al., 1990). Vn is a glycoprotein which is synthesized in the liver and circulates in plasma at high concentration (200–400 μ g/ml). The insoluble form is localized extravascularly and is associated with granulation tissue areas in rheumatoid arthritis synovia (Seiffert et al., 1993).

It has been reported that β_3 complex signaling via CD47 affected β_2 (CD18/CD11b and CD18/CD11c) integrins binding to their ligands (Van Strijp et al., 1993; Ishibashi et al., 1994). Previous studies identified CD11b and CD11c as novel ligands for sCD23 (Lecoanet-Henchoz et al.,

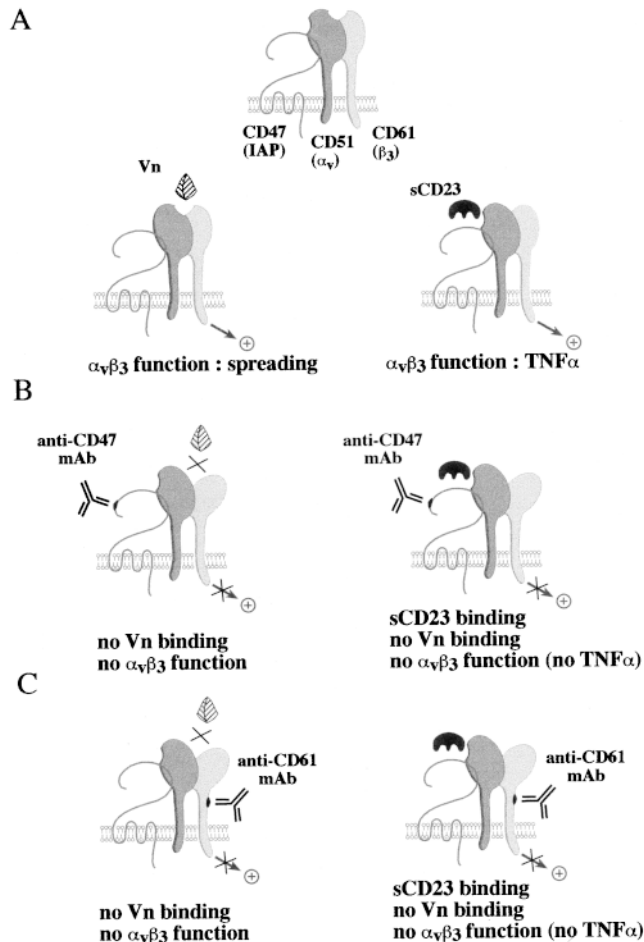


Figure 9. sCD23 and Vn share the same functional receptor, VnR/CD47 complex. (A) sCD23 binds to α_v /CD51 and Vn to $\alpha_v\beta_3$ (CD51/CD61) conformational site while neither bind to CD47. Binding of Vn to $\alpha_v\beta_3$ (CD51/CD61) obliterates sCD23 binding site, and vice versa. (B and C) Anti-CD47 and anti- β_3 (CD61) mAbs suppress Vn binding and function (left) while they inhibit sCD23 function, but not binding (right).

1995). Our study shows that sCD23 bound CD11⁻ cell lines (Jurkat and CHO cells; Figs. 1 and 8) failed to stain the THP-1 (CD11⁺) cell line (Fig. 8), and anti-CD47 mAb did not alter CD11b expression, or sCD23 binding on monocytes (data not shown), indicating that sCD23 does not interact with β_2 integrins. We currently have no explanation for these contradictory results.

Interestingly, our unpublished data indicating TSP, a newly discovered CD47 ligand (Gao et al., 1996b), also suppressed sCD23 function, without directly triggering monokine release, further supporting our present model. The absence of VnR-mediated monokine secretion following engagement by Vn, Fn, or TSP does not exclude the possibility that other ligands (see review Felding-Habermann and Cheresh, 1993; Gladson and Cheresh, 1994) would share, with sCD23, its proinflammatory activity. Finally, the ability of the anti-CD47 mAbs examined to inhibit the function of sCD23 without interfering with the phagocytosis of senescent cells (Savill et al., 1990) may help in the design of novel therapeutic strategies for chronic inflam-

matory disorders, such as rheumatoid arthritis, in which CD23 and TNF- α are implicated (Plater-Zyberk and Bonnefoy, 1995; Feldman et al., 1996).

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