Brief Definitive Report

CD47 Ligation Selectively Downregulates Human Interleukin 12 Production

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Summary

Interleukin (IL)-12 plays a key role not only in protective innate and adaptive T helper cell type 1 (Th1) responses but also in chronic inflammatory diseases. We report here that engagement of CD47 by either monoclonal antibody, its natural ligand thrombospondin (TSP), or 4N1K (a peptide of the COOH-terminal domain of TSP selectively binding CD47) inhibits IL-12 release by monocytes. The suppression occurred after T cell–dependent or –independent stimulation of monocytes and was selective for IL-12 inasmuch as the production of tumor necrosis factor (TNF)-α, IL-1, IL-6, and granulocyte/macrophage colony-stimulating factor was not inhibited. CD47 ligation did not alter transforming growth factor (TGF)-β and IL-10 production, and the suppressive effect on IL-12 was not due to autocrine secretion of TGF-β or IL-10. The IL-12 inhibition was not mediated by Fcγ receptor ligation, did not require extracellular Ca^{2+} influx, but was reversed by two phosphoinositide 3-kinase inhibitors (wortmannin and Ly294002). Thus, engagement of CD47 on monocytes by TSP, which transiently accumulates at the inflammatory site, is a novel and unexplored pathway to selectively downregulate IL-12 response. The pathway may be relevant in limiting the duration and intensity of the inflammatory response, and in developing novel therapeutic strategies for Th1-mediated diseases.

Key words: CD47 • interleukin 12 • monocytes • thrombospondin

Interleukin (IL)-12 is a potent proinflammatory and immunoregulatory cytokine which plays a crucial role in innate and adaptive immunity (for a review, see reference 1). IL-12 is produced primarily by activated mononuclear and polynuclear phagocytes, as well as dendritic cells. IL-12 is released during the early stage of infections caused by a large variety of bacteria, intracellular pathogens, fungi, and certain viruses. This early production of IL-12 is T cell independent and results from the direct interaction of pathogens or their products with phagocytic cells. IL-12 rapidly triggers NK cells and T cells to release IFN-γ, which increases not only the bactericidal activity of phagocytes but also their release of proinflammatory cytokines, including IL-12 itself. In addition to activating the cells of the innate immune system, IL-12 and IFN-γ direct the differentiation of antigen-specific naive T cells into Th1 effectors, producing high levels of IFN-γ and conferring protection against intracellular pathogens.

The IL-12/IFN-γ proinflammatory loop initiated during the early stage of infection is generally of short duration and is probably downregulated by several mechanisms. For example, the engagement of phagocytic receptors (CR3, Fcγ, and scavenger receptors) on macrophages by opsonized bacteria was shown to inhibit IL-12 production (2, 3). Similarly, CD46 ligation by measle virus suppresses IL-12 release (4). Another important physiological inhibitor of IL-12/IFN-γ production is IL-10, which can also be released by pathogen-stimulated phagocytes, but at a later stage than IL-12 (1). Other nonselective inhibitors of IL-12 production include cytokines like TGF-β, IL-4, and IL-13, as well as PGE2, glucocorticoids, and cholera toxin (5–7).

IL-12 is also produced after cognate interaction of $CD4^+$ T cells with dendritic cells or monocytes (8). This T cell–dependent pathway of IL-12 production is mediated through CD40–CD154 (CD40L) interactions and regulates the level of IFN-γ production in primary and secondary response to antigens devoid of intrinsic adjuvant activity, including autoantigens (1). Recent evidence further suggests a third mechanism of IL-12 production which may occur at inflammatory sites, without infectious agents or TCR-mediated signals (9). It was found that the addition of IL-15 or IL-2 to cultures of autologous resting T cells and monocytes induces the production of proinflammatory cytokines including IL-12 and IFN-γ.

The extravasation of T lymphocytes and monocytes from blood vessels to inflammatory lesions requires interactions with the extracellular matrix (ECM), which is composed of various adhesive glycoproteins and glycosamino-

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glycans (10). Consequently, molecules that mediate the physical interactions between the immune cells and the extracellular environment may play a critical role by imposing a context in which biological and inflammatory processes take place. For instance, low molecular weight hyaluronan was shown to induce IL-12 production by binding CD44 on inflamed macrophages (11). Also, the β1 and β2 integrin family of adhesive receptors, expressed by PBMCs, mediates ECM–cell interactions that deliver costimulatory signals to the incoming cells. Engagement of β1 integrins by fibronectin increases adhesion of T lymphocytes to immobilized ligands (12) and promotes TCR signaling.

CD47 antigen, also known as integrin-associated protein (IAP), is a widely expressed multispan transmembrane protein which is physically and functionally associated with αvβ3 integrin, the vitronectin (Vn) receptor (13). Indeed, CD47$^-$ cell lines expressed αvβ3 but failed to bind Vn-coated beads. Moreover, CD47-deficient mice rapidly die of *Escherichia coli* peritonitis, a phenomenon directly associated with a reduction in leukocyte activation in response to β3, but not β2, integrin ligation (14). More recently, CD47 has been implicated in leukocyte transendothelial migration (15), whereas its potential role in immune regulation has not yet been explored. Its newly described natural ligand, thrombospondin (TSP), is transiently expressed at high concentration in damaged and inflamed tissues (16, 17). TSP is a homotrimeric ECM protein mainly produced by platelets; it may also be synthesized by monocytes and alveolar macrophages. In addition to CD47, TSP binds heparan sulfate proteoglycans, the αvβ3, α4β1, α5β1 integrins, and CD36 (16–18).

We here examine the potential role of CD47 and TSP in the regulation of cytokine synthesis by monocytes. We report that ligation of CD47 antigen, by mAb or TSP, specifically and selectively inhibits IL-12 production by purified monocytes in response to T cell–dependent or –independent stimuli.

Materials and Methods

Reagents. Recombinant GM-CSF was provided by Dr. D. Bron (Institut Bordet, Brussels, Belgium). IFN-γ was obtained from Genzyme. sCD40L trimeric protein was provided by Drs. C. Maliszewski and R. Armitage (Immunex Corp., Seattle, WA). *Staphylococcus aureus* Cowan I strain (SAC) was used at 0.01% (wt/vol) (Pansorbin; Calbiochem-Behring) and LPS at 10 μg/ml (Sigma Chemical Co.). Anti-CD47 mAbs B6H12 (mouse IgG1) and BRIC126 (mouse IgG2b) were purchased from Serotec. Isotype-matched negative control mAb (mouse IgG1) was prepared in our laboratory. Anti-CD18 mAb (mouse IgG1; American Type Culture Collection) was used at 10 μg/ml. Neutralizing anti–IL-10 mAb (clone 19 F1.1; American Type Culture Collection) and neutralizing anti–TGF-β (polyclonal chicken Ig) were used at 10 and 30 μg/ml, respectively. TSP was purchased from GIBCO BRL, and EDTA and EGTA were from Sigma Chemical Co. The 4N1K peptide (KRFYVVMWKK) was obtained from Genosys and corresponds to the COOH-terminal domain of TSP (16); 4NGG (KRFYGGMWKK) was a gift from W. Frazier (Washington University, St. Louis, MO).

Cell Purification and Culture Conditions: Monocytes. PBMCs were isolated by density gradient centrifugation of heparinized blood from healthy volunteers (total $n = 30$) using Lymphoprep (Nycomed). Enriched monocytes were prepared by cold aggregation as described (9), followed by one cycle of rosetting with S-(2-aminoethyl) isothiouronium bromide (Aldrich)–treated SRBCs to deplete residual T and NK cells. Monocyte purity was shown to be >95% by flow cytometry (FACScan™; Becton Dickinson) using PE-conjugated anti-CD14 mAb (Ancell).

Monocytes were cultured at 10^6/ml in 96-well round-bottomed Falcon plates (Becton Dickinson). Cultures were performed in quadruplicate in complete serum-free HB101 medium (Irvine Scientific) containing Ca^{2+} (600 μM) and Mg^{2+} (490 μM) and supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 10 mM Hepes, 100 IU penicillin, and 100 μg/ml streptomycin.

Cytokine Measurement. IL-12p70 release was assessed by a two-site sandwich ELISA using clone 20C2 as the capture mAb and clone 4D6 as the second mAb as described (9). Both mAbs were provided by Dr. M. Gately (Hoffmann-LaRoche, Nutley, NJ). The sensitivity of the assay was 6 pg/ml. TNF-α was measured using a sandwich ELISA as described previously (19), and IL-10 was determined by a sandwich solid-phase RIA using anti–IL-10 mAb (clone 9D7) as the capture mAb and ^{125}I-labeled anti–IL-10 (clone 12G8) as the detecting probe. IL-1β, IL-6, GM-CSF, and TGF-β ELISA kits were purchased from R&D Systems. All of the measurements were performed in duplicate with a coefficient variation of <10%.

Statistical Analysis. The paired *t* test was used to determine statistical significance of the data. $*P < 0.05$; $**P < 0.01$; $***P < 0.005$.

Results

CD47 mAb Selectively Suppresses IL-12 Release by Monocytes. We examined the effect of soluble CD47 mAb on IL-12 release by purified monocytes costimulated by IFN-γ and T cell–dependent (sCD40L and GM-CSF) or –independent (SAC) signals. As depicted in Fig. 1 A, CD47 mAb significantly suppressed IL-12 secretion in response to either stimuli, whereas isotype-matched IgG1 mouse mAb had no effect. In agreement with Marth and Kelsall (3), anti-CD18 mAb, used as a cell-binding irrelevant mAb, did not suppress IL-12 production (data not shown). We noted an inhibition of IL-12 release even when monocytes were cultured with SAC alone, indicating that CD47 mAb did not simply impair the enhancing effect of IFN-γ on IL-12 production. Our unpublished observations revealed a similar suppressive effect by CD47 mAb after LPS and IFN-γ costimulation.

The blockade of IL-12 production in response to SAC and IFN-γ was rather selective, since the release of other monocyte products (TNF-α, IL-1, IL-6, and GM-CSF) remained largely unaffected (Fig. 1 B). Note a threefold increase in IL-6 secretion, which did not reach statistical significance. Cell viability, as determined by trypan blue dye exclusion, was unchanged. Also, the inhibition of IL-12 was probably not mediated via an increase of autocrine antiinflammatory cytokines (IL-10 and TGF-β), known to efficiently downregulate bacteria-induced monokine release (1). As shown in Fig. 1 C, IL-10 and TGF-β levels remained unaffected by CD47 mAb treatment. Moreover, neutralizing

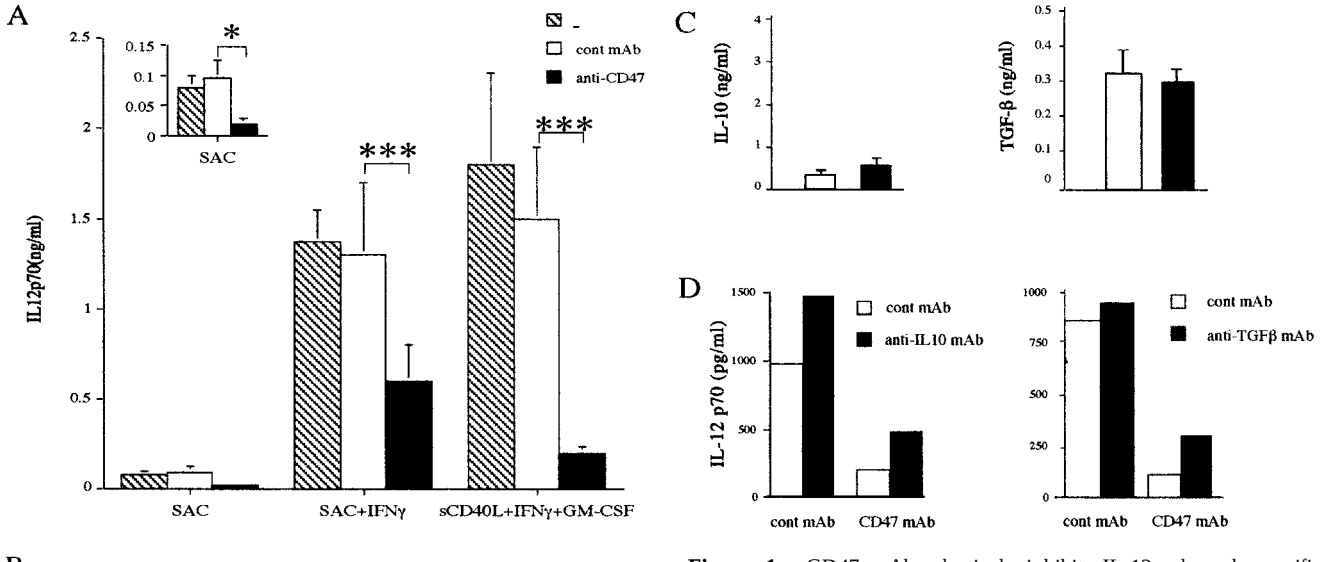

Figure 1. CD47 mAb selectively inhibits IL-12 release by purified monocytes. (A) Monocytes (10^6/ml) were cultured with SAC (0.01%; $n = 5$), SAC plus IFN-γ (500 U/ml; $n = 12$), or sCD40L (1 μg/ml) plus GM-CSF (25 ng/ml) plus IFN-γ (500 U/ml; $n = 6$) in the absence or presence of CD47 mAb (IgG1) or isotype-matched control mAb (cont; 10 μg/ml). IL-12p70 was measured in the culture supernatants after 20 h. (B) Monocytes were stimulated with SAC and IFN-γ ($n = 6$) in the presence of CD47 or control mAb. TNF-α, IL-1β, IL-6, and GM-CSF were measured after 20 h. Data are mean values ± SEM of n separate experiments from n different donors. $^{***}P < 0.005$; $^{*}P < 0.05$. (C) Monocytes were stimulated with SAC and IFN-γ in the presence of CD47 or control mAb; IL-10 and TGF-β were measured after 20 h. Data are mean values ± SEM from six experiments using six different donors. (D) Monocytes were stimulated with SAC and IFN-γ in the presence of CD47 or control mAb; neutralizing anti–IL-10 (10 μg/ml), anti–TGF-β (30 μg), or isotype-matched control mAbs were added to the cultures. Data are mean values of duplicate cultures and represent one of three experiments using three different donors.

anti–IL-10 or anti–TGF-β antibodies added alone or simultaneously did not overcome CD47 mAb–mediated inhibition of IL-12 production by SAC- and IFN-γ–stimulated monocytes (Fig. 1 D, and data not shown).

Thrombospondin Inhibits IL-12 Release. We next evaluated the effect of TSP, the natural ligand of CD47 (16), on monokine release. As shown in Table I, TSP significantly reduced IL-12 production by SAC- and IFN-γ–stimulated monocytes, whereas it slightly enhanced TNF-α release. Note that monocytes coexpressed two other TSP receptors (CD36 and αvβ3 [15]). Since the site of interaction between CD47 and TSP is limited to the cell-binding domain (CBD) of TSP, we examined the role of a synthetic peptide (4N1K) encoding this particular domain (16). We found that 4N1K, but not control mutant peptide 4NNG, dose-dependently inhibited IL-12 release in response to SAC and IFN-γ without affecting TNF-α production (Fig. 2).

CD47 Ligation Suppresses IL-12 Release via an Fc and Ca^{2+}–independent but Phosphoinositide 3-Kinase–dependent Pathway. This specific inhibition of IL-12 release after CD47 engagement was reminiscent of recent studies reporting that FcγR cross-linking selectively downregulated IL-12 secretion in monocytes via an increase in extracellular calcium influx (2). We analyzed whether this mechanism might be involved in the CD47 mAb–mediated IL-12 suppression. First, $F(ab')_2$ fragments of CD47 mAb dose-dependently inhibited IL-12p70 release, demonstrating that the inhibitory effect was not Fc mediated (Fig. 3 A). Furthermore, monovalent Fab fragments still suppressed IL-12 secretion (Fig. 3 B), suggesting that CD47 mAb either prevented monocyte/monocyte interactions and the delivery of a positive signal, or directly delivered to the monocytes a negative signal for IL-12 production. Second, IL-12 downregulation was observed in monocyte cultures supplemented with a fivefold excess of the Ca^{2+} and Mg^{2+} chelating agents EDTA or EGTA, demonstrating that the suppressive effect was independent of extracellular Ca^{2+} influx (Fig. 3 B).

Table I. *TSP Selectively Inhibits IL-12 Production by Monocytes*

	IL-12 (pg/ml)			TNF-α (ng/ml)		
	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
SAC + IFN-γ	168	1,020	2,120	120	64	87
SAC + IFN-γ + TSP	79	746	1,090	147	83	105
Percent inhibition	53	27	49	–	–	–

Monocytes (10^6/ml) were cultured for 20 h with SAC (1:10,000) and IFN-γ (500 U/ml) with or without plastic-coated TSP (10 μg/ml). Culture supernatants were harvested for cytokine determination as described. Exp., experiment.

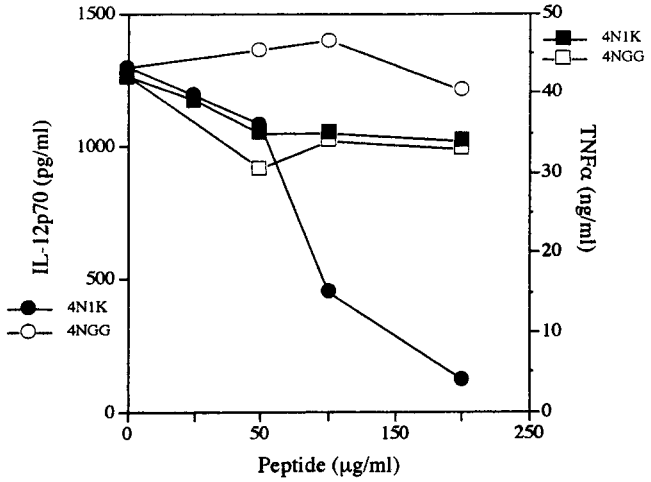

Figure 2. 4N1K selectively suppresses the production of IL-12 by monocytes. Monocytes (10^6/ml) were stimulated with SAC and IFN-γ in the presence of various concentrations of 4N1K (peptide of COOH-terminal domain of TSP) or 4NGG (mutant peptide). IL-12p70 and TNF-α release were measured after 20 h. Data are mean values of duplicate cultures of one representative of three independent experiments using three different donors.

Moreover, addition of EDTA or EGTA in the absence of mAb did not result in inhibition of TNF-α or IL-12 release.

Since TSP signaling of focal adhesion in endothelial cells requires activation of phosphoinositide 3-kinase (PI 3-K [20]), we explored the involvement of this pathway in CD47 mAb–mediated suppression of IL-12 release by using two PI 3-K inhibitors, wortmannin and Ly294002. We found that both PI 3-K inhibitors restored IL-12 release in a dose-dependent manner (Fig. 4) with no effect on TNF-α secretion (data not shown). However, neither protein kinase C (e.g., H7, HA1004) nor protein tyrosine kinase inhibitors (e.g., herbimycin) prevented CD47 mAb–mediated inhibition of IL-12 release (not shown). Taken collectively, our results suggest that engagement of CD47 on monocytes by mAb, its natural ligand TSP, and 4N1K peptide specifically suppressed IL-12 secretion through an Fc and Ca^{2+}–independent but PI 3-K–dependent pathway.

Discussion

The critical role of IL-12–mediated protection against mycobacterial infection was recently confirmed in IL-12 receptor–deficient patients (21). Nevertheless, uncontrolled production of IL-12 is pathogenic in some organ-specific autoimmune diseases (1) and endotoxic shock (22), underscoring the requirement of potent negative regulatory feedback mechanisms. The present findings demonstrate that engagement of CD47 by its natural ligand, TSP, or by CD47 mAb, represents a novel pathway to selectively downregulate IL-12 production.

Inhibition of IL-12 release by CD47 ligation occurred in activated monocytes through a PI 3-K–dependent pathway. Indeed, two specific inhibitors of PI 3-K (wortmannin and Ly294002) overcame CD47-mediated IL-12 suppression with no effect on TNF-α production. Although these data are not a definite proof of the involvement of PI 3-K, they are supported by the observations that TSP-mediated functions such as cell spreading or focal adhesion disassembly required activation of PI 3-K (20). Besides inhibitory cytokines, including IL-4, IL-10, and TGF-β, which suppress the production of a large number of proinflammatory products (e.g., TNF-α, IL-1, IL-6, and IL-12) by activated monocytes, several monocyte surface receptors reportedly downregulate IL-12 production in a somewhat selective manner. *Histoplasma capsulatum* and measles virus have been shown to decrease IL-12 release by signaling through CD11b and CD46, respectively (3, 4). IL-12 downmodulation that resulted from scavenger receptor and FcγR ligation is due to extracellular Ca^{2+} influx (2) and increase in IL-10 production in the case of FcγRI (23). In the present study, the CD47 mAb inhibition was Fc and Ca^{2+} independent since intact, divalent, and monovalent fragments of CD47 mAb

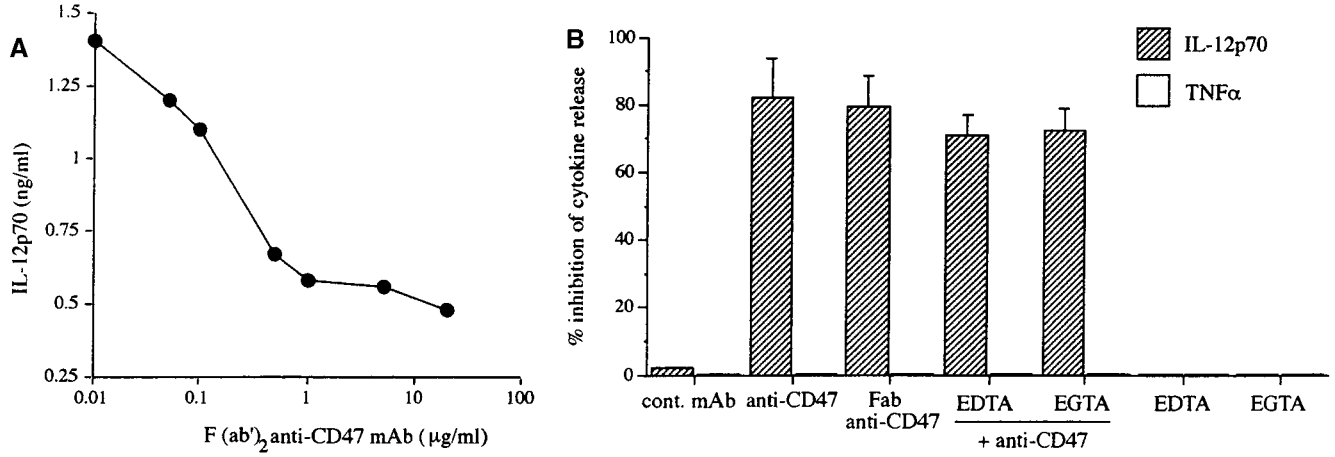

Figure 3. Suppression of IL-12 production by CD47 mAb is Fc and Ca^{2+} independent. Monocytes (10^6/ml) were stimulated with SAC and IFN-γ and titrated concentrations of $F(ab')_2$ anti-CD47 mAb (A) or 20 μg/ml of Fab anti-CD47, EDTA, and EGTA (5 mM) (B). IL-12p70 and TNF-α release were measured after 20 h. Results in A represent mean values of one out of three separate experiments using three different donors. Results in B are expressed as means ± SD of percent inhibition of control response (SAC- and IFN-γ–treated monocytes) of four separate experiments using four different donors.

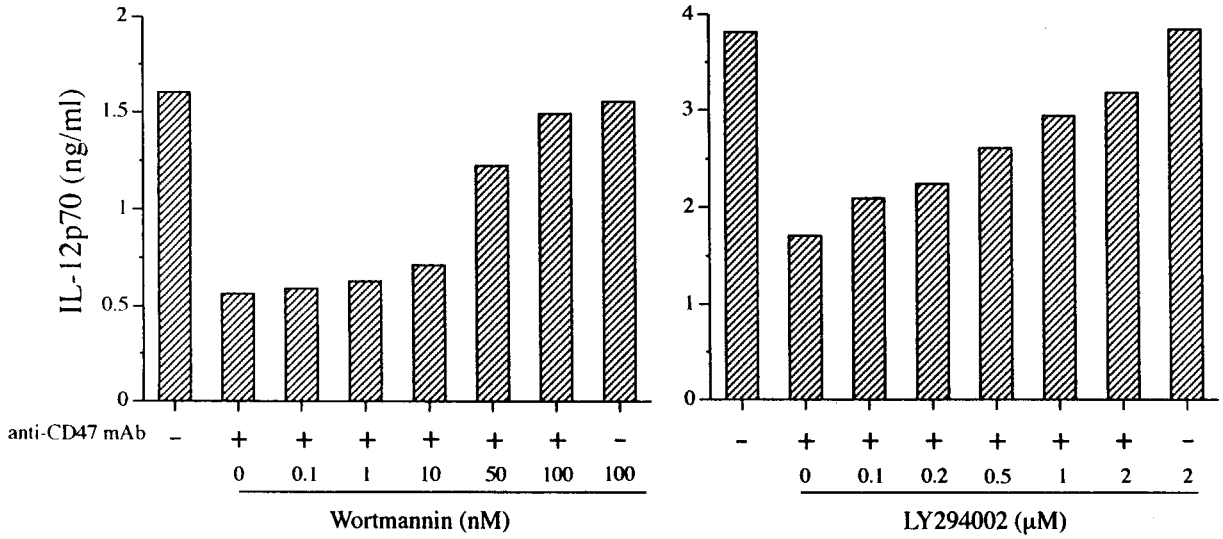

Figure 4. PI 3-K inhibitors restore IL-12 production. Monocytes (10^6/ml) were stimulated with SAC and IFN-γ in the absence or presence of CD47 mAb (10 μg/ml) and titrated concentrations of wortmannin or Ly294002. IL-12p70 release was measured after 20 h. Results represent mean values from one of three independent experiments using three different donors.

suppressed IL-12 regardless of the presence of divalent cation chelating agents.

CD47 antigen, also named integrin-associated protein (IAP), regulates the function and the binding of Vn to αvβ3, its associated integrin (13). However, Vn does not bind CD47, and the natural ligand of CD47 is reported to be TSP (16). TSP interacts with CD47 through its CBD, but binds other surface receptors such as CD36 or αvβ3 via distinct domains (17, 24).

Since TSP reportedly activates latent TGF-β in vitro and in vivo (25) and ligation of CD36 by CD36 mAb downregulates IL-12 and TNF-α production and increases IL-10 (26), we used 4N1K peptide encoding the CBD of TSP to demonstrate that TSP-mediated inhibition of IL-12 resulted from its direct effect on CD47 antigen. In addition, CD47 ligation did not decrease TNF-α nor increase IL-10 production. Note that TSP uses its properdin-like domain to activate TGF-β (17) and that TGF-β production was not upregulated after CD47 signaling (Fig. 1 C).

IL-12 downmodulation, in the absence of TNF-α inhibition, was also observed in IFN regulatory factor (IRF-1 [27]) and IFN consensus sequence binding protein (ICSBP)-deficient mice (28). These two transcription factors belong to the IRF family and bind IL-12p40 promoter as well as other IFN-stimulated genes. As a direct consequence of the primary defect in IL-12 production, the deficient mice displayed an impaired Th1 response and IFN-γ–dependent host resistance to intracellular pathogens. Similarly, our unpublished observations indicate that CD47 signaling strongly impaired IL-12–induced proliferative and IFN-γ responses of activated T cells. The role of CD47 antigen, which is expressed by all leukocytes, has been demonstrated in the transendothelial migration of neutrophils, lymphocytes, and more recently, monocytes (15). Our present findings reveal that CD47 ligation by TSP modulates the function of monocytes. Therefore, we propose that engagement of CD47 is a novel and unexplored pathway to selectively downregulate IL-12 production. Inhibition of the IL-12 response is crucial for the treatment of organ-specific autoimmune diseases (1). For instance, administration of anti–IL-12 mAb completely abrogated established colitis of trinitrobenzene sulfonic acid (TNBS)-treated mice. IL-12 neutralization was also revealed to be an effective treatment of autoimmune encephalitis and insulin-dependent diabetes.

Finally, in addition to its ability to downregulate IL-12 response through its CBD, TSP is known to display several important antiinflammatory activities via distinct domains. TSP facilitates elimination of apoptotic cells (29), activates TGF-β in vivo and in vitro (25), potently inhibits angiogenesis (17), and suppresses HIV infectivity through its binding to HIVg120 (24). Moreover, absence of TSP causes pneumonia (30). Therefore, since TSP is transiently expressed at high concentration in damaged and inflamed tissue, its selective suppression of the IL-12 response through interaction with the CD47 molecule may be relevant in limiting the duration and the intensity of the inflammatory response. The importance of such a process awaits confirmation in CD47- and TSP-deficient mice.

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