CD47 Ligation Selectively Downregulates Human Interleukin 12 Production

By Myriam Armant, Marie-Noëlle Avice, Patrice Hermann, Manuel Rubio, Mamoru Kiniwa, Guy Delespesse, and Marika Sarfati

From Laboratoire d’Immunologie, Centre de Recherche du Centre Hospitalier de l’Université de Montréal (CHUM), Campus Notre-Dame, Quebec H2L 4M1, Canada

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 Ca²⁺ 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
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 E. 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. (Sigma Chemical Co.). The 4N1K peptide (KRFYVVMWKK) was obtained from Genosys and corresponds to the COOH-terminal domain of TSP (16); 4N GG (KRFY GGMMWKK) 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 (Alldrich)-treated SR BCs 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 (Ancll).

Monocytes were cultured at 10% ml in 96-well round-bottomed Falcon plates (Becton Dickinson). Cultures were performed in quadruplicate in complete serum-free H1B101 medium (Irvin Scientific) containing Ca2+ (600 μM) and Mg2+ (490 μM) and supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 10 mM Hepes, 100 IU penicillin, and 100 μg/ml streptomycin. Cytokine measurements. 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-La Roche, 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 125I-labeled anti-IL-10 (clone 12G6) as the detecting probe. IL-1p, 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 of 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 stimulus, 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).
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) |
|----------------|--------------|--------------|
| SAC + IFN-γ    | 168          | 1,020        |
| SAC + IFN-γ + TSP | 79          | 746          | 1,090 |
| Percent inhibition | 53          | 27           | 49    |

M 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.
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 Ca²⁺–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) overcome 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 FcγRI ligation is due to extracellular Ca²⁺ 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 Ca²⁺ independent since intact, divalent, and monovalent fragments of CD47 mAb...
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 \( \alpha v \beta 3 \), 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 \( \alpha v \beta 3 \) via distinct domains (17, 24).

Since TSP reportedly activates latent TGF-\( \beta \) in vitro and in vivo (25) and ligation of CD36 by CD36 mAb downregulates IL-12 and TNF-\( \alpha \) 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-\( \alpha \) nor increase IL-10 production. Note that TSP uses its properdin-like domain to activate TGF-\( \beta \) (17) and that TGF-\( \beta \) production was not upregulated after CD47 signaling (Fig. 1C).

IL-12 downmodulation, in the absence of TNF-\( \alpha \) 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-\( \gamma \)-dependent host resistance to intracellular pathogens. Similarly, our unpublished observations indicate that CD47 signaling strongly impaired IL-12-induced proliferative and IFN-\( \gamma \) 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-\( \beta \) in vivo and in vitro (25), potently inhibits angiogenesis (17), and suppresses HIV infectivity through its binding to H1Vg120 (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.

Figure 4. PI 3-K inhibitors restore IL-12 production. Monocytes (10^6/ml) were stimulated with SAC and IFN-\( \gamma \) in the absence or presence of CD47 mAb (10 \( \mu g \)/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.
References

1. Gately, M.K., L.M. Renzetti, J. Magram, A.S. Stern, L. Adorini, U. Gubler, and D.H. Presky. 1998. The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. Annu. Rev. Immunol. 16: 495–521.

2. Sutterwala, F.S., G.J. Noel, R. Clynes, and D.M. Mosser. 1997. Selective suppression of interleukin-12 induction after macrophage receptor ligation. J. Exp. Med. 185:1977–1985.

3. Mirth, T., and B.L. Kelsall. 1997. Regulation of interleukin-12 by complement receptor 3 signaling. J. Exp. Med. 185: 1987–1995.

4. Karp, C.L., M. Wysocka, L.M. Wahl, P.J. Cuomo, B. Sherry, G. Trinchieri, and D.E. Griffin. 1996. Mechanism of suppression of cell-mediated immunity by measles virus. Science. 273:228–231.

5. van der Pouw Kraan, T.C., L.C. Boeije, R.J. Smeenk, J. Vijdens, and L.A. Arden. 1995. Prostaglandin-E2 is a potent inhibitor of human interleukin 12 production. J. Exp. Med. 181:775–779.

6. Blotta, M.H., R.H. DeKruyff, and D.T. U metsu. 1997. Corticosteroids inhibit IL-12 production in human monocytes and enhance their capacity to induce IL-4 synthesis in CD4+ lymphocytes. J. Immunol. 158:5589–5595.

7. Braun, M.C., J. He, C.-Y. Wu, and B.L. Kelsall. 1999. Cholera toxin suppresses interleukin-12 production and IL-12 receptor β1 and β2 chain expression. J. Exp. Med. 189:541–552.

8. Shu, U., M. Kiniwa, C.Y. Wu, C.M. Aliszewski, N. Vezzio, J. Hakimi, M. Gately, and G. Deleespse. 1995. Activated T cells induce interleukin-12 production by monocytes via CD40–CD40 ligand interaction. Eur. J. Immunol. 25:1125–1128.

9. Avice, M.-N., C.E. Demetre, G. Deleespe, M. Rubio, M. Arman t, and M. Sarfati. 1998. IL-15 promotes IL-12 production by human monocytes via T cell-dependent contact and may contribute to IL-12-mediated IFN-γ secretion by CD4+ T cells in the absence of TCR ligation. J. Immunol. 161:3408–3415.

10. Ben-Baruch, A., D.F. Michiel, and J.J. Oppenheim. 1995. Signals and receptors involved in recruitment of inflammatory cells. J. Biol. Chem. 270:11703–11706.

11. Hodge-Dufour, J., P.W. Noble, M.R. Horton, C. Bao, M. Wysocka, M.D. Burdick, R.M. Strieter, G. Trinchieri, and E. Puré. 1997. Induction of IL-12 and chemokines by hyaluronan requires adhesion-dependent priming of resident but not elicited macrophages. J. Immunol. 159:2492–2500.

12. Aguirre, J.E., K.M. Danahay, L.C. Burkly, and G.A. van Seventer. 1995. T cell receptor– and β1 integrin-mediated signals synergize to induce tyrosine phosphorylation of focal adhesion kinase (pp125FAK) in human T cells. J. Exp. Med. 182:2079–2090.

13. Lindberg, F.P., H.D. Gresham, E. Schwarz, and E.J. Brown. 1993. Molecular cloning of integrin-associated protein: an immunoglobulin family member with multiple membrane-spanning domains implicated in αvβ3-dependent ligand binding. J. Cell Biol. 123:485–496.

14. Lin dberg, F.P., D.C. Bullard, T.E. Caver, H.D. Gresham, A.L. Beaudet, and E.J. Brown. 1996. Decreased resistance to bacterial infection and granulocyte defects in IL-12-deficient mice. Science. 274:795–798.

15. Weera singhe, D., K.P. M Ch ugh, F.P. Ross, E.J. Brown, R.H. Gisler, and B.A. Imhof. 1998. A role for the αvβ3 integrin in the transmigration of monocytes. J. Cell Biol. 142: 595–607.

16. Gao, A.-G., F.P. Lindberg, M.B. Finn, S.D. Blystone, E.J. Brown, and W.A. Fra zier. 1996. Integrin-associated protein is a receptor for the C-terminal domain of thrombospondin. J. Biol. Chem. 271:21–24.

17. Bornstein, P. 1992. Thrombospondins: structure and regulation of expression. FASEB J. 6:3290–3299.

18. Yakowitz, R., V.M. Dixit, N. Guo, D.D. Roberts, and Y. Shimizu. 1993. Activated T-cell adhesion to thrombospondin is mediated by the α4β1 (VLA-4) and α5β1 (VLA-5) integrins. J. Immunol. 151:149–158.

19. Arman t, M., H. Ishihara, M. Rubio, G. Deleespse, and M. Sarfati. 1994. Regulation of cytokine production by soluble CD23: costimulation of interferon-γ secretion and triggering of tumor necrosis factor α release. J. Exp. Med. 180:1005–1011.

20. Greenwood, J.A., M.A. Pallero, A.B. Theibert, and J.E. Murphy-Ullrich. 1998. Thrombospondin signaling of focal adhesion disassembly requires activation of phosphoinositide 3-kinase. J. Biol. Chem. 273:1755–1763.

21. Altare, F., A. Durandy, L. Lammas, J.-F. Emile, S. Lamhamedi, F. Le Deist, P. Drysdale, E. Jouanguy, R. Döffinger, F. Bernaudin, et al. 1998. Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency. Science. 280:1432–1438.

22. Wysocka, M., M. Kubin, L.Q. Vieira, L. Ozmen, G. Garotta, P. Scott, and G. Trinchieri. 1995. Interleukin-12 is required for interferon-γ production and lethality in lipopolysaccharide-induced shock in mice. Eur. J. Immunol. 25:672–676.

23. Sutterwala, F.S., G.J. Noel, P. Salgame, and D.M. Mosser. 1998. Reversal of proinflammatory responses by ligating the macrophage Fcγ receptor type 1. J. Exp. Med. 188:217–222.

24. Crombie, R., R.L. Silverstein, C. MacLow, S.F.A. Pearce, R.L. N achman, and J. Laurence. 1998. Identification of a CD36-related thrombospondin 1–binding domain in HIV-1 envelope glycoprotein gp120: relationship to HIV–1–specific inhibitory factors in human saliva. J. Exp. Med. 187:25–35.
25. Crawford, S.E., V. Stellmach, J.E. Murphy-Ullrich, S.M.F. Ribeiro, J. Lawler, R.O. Hynes, G.P. Boivin, and N. Bouck. 1998. Thrombospondin-1 is a major activator of TGF-β1 in vivo. Cell. 93:1159–1170.
26. Voll, R.E., M. Herrmann, E.A. Roth, C. Stach, and J.R. Kalden. 1997. Immunosuppressive effects of apoptotic cells. Nature. 390:350–351.
27. Lohoff, M., D. Ferrick, H.-W. Mittrücker, G.S. Duncan, S. Bischof, M. Röllinghoff, and T.W. Mak. 1997. Interferon regulatory factor-1 is required for a T helper 1 immune response in vivo. Immunity. 6:681–689.
28. Scharton-Kersten, T., C. Contursi, A. Masumi, A. Sher, and K. Ozato. 1997. Interferon consensus sequence binding protein-deficient mice display impaired resistance to intracellular infection due to a primary defect in interleukin 12 p40 induction. J. Exp. Med. 186:1523–1534.
29. Ren, Y., and J. Savill. 1995. Proinflammatory cytokines potentiate thrombospondin-mediated phagocytosis of neutrophils undergoing apoptosis. J. Immunol. 154:2366–2374.
30. Lawler, J., M. Sunday, V. Thibert, M. Duquette, E.L. George, H. Rayburn, and R.O. Hynes. 1998. Thrombospondin-1 is required for normal murine pulmonary homeostasis and its absence causes pneumonia. J. Clin. Invest. 101:982–992.