Regulation of Interleukin-12 by Complement Receptor 3 Signaling

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Summary
Complement receptor type 3 (CR3, CD11b/CD18) serves as a receptor for a number of endogenous ligands and infectious organisms, and is involved in adhesion and host defense functions. Here, we report that signaling via CR3 plays an important role in regulating production of interleukin-12 (IL-12), a key mediator of cell-mediated immunity (CMI). We demonstrate with a variety of stimuli a dose-dependent, specific downregulation of IL-12 secretion by human monocytes in vitro after exposure to antibodies to CR3 (anti-CD11b and anti-CD18), as well as to the natural CR3 ligands, iC3b, and Histoplasma capsulatum. CR3 antibodies also suppressed interferon-γ (IFN-γ) production in cultures of human peripheral blood mononuclear cells (PBMC). We determined that one mechanism by which CR3 antibodies may suppress IL-12 production is by the inhibition of IFN-γ-induced tyrosine phosphorylation. Finally, in a murine model of IL-12-dependent septic shock, we provide evidence that administration of CR3 antibodies leads to suppression of IL-12 and IFN-γ in vivo. Our studies thus define a novel role for CR3 in regulating CMI functions via IL-12.

CR3 is a heterodimeric molecule which, like LFA-1 (CD11a/CD18) and CR4 (CD11c/CD18), belongs to the β2-integrin family of cell adhesion molecules (1-5). CR3 is expressed mainly on polymorphonuclear leukocytes, monocytes and macrophages, and natural killer cells (1, 2), and interacts with a variety of ligands including complement fragment iC3b, intercellular adhesion molecule 1 (ICAM-1), fibrinogen, and β-glucan (2, 4). Moreover, it mediates the binding of opsonized or unopsonized infectious agents such as Histoplasma capsulatum, Leishmania major, group B streptococci, Bordetella pertussis, Candida albicans, and several mycobacteria (2, 5-7).

Previous studies have shown that CR3 is involved in several monocyte and macrophage functions including transmigrational adhesion (2, 4, 8), phagocytosis, nitric oxide production and the generation of a respiratory burst (2, 3, 5, 8, 10). In addition, CR3 signaling may indirectly affect T cell function as the administration of antibodies to CR3 to animals suppresses delayed type hypersensitivity (DTH) reactions (11, 12), and fatally potentiates infections with Listeria monocytogenes or Toxoplasma gondii (13, 14). Since these phenomena are dependent on an intact cell-mediated immunity (CMI) and Th1 responses, we explored the role of CR3 signaling in regulating cytokines that mediate such responses.

Materials and Methods
Cell Isolation, Cell Culture Conditions and Assessment of Cytokine Production. Human monocytes were obtained from healthy donors (total n = 14) by standard leukapheresis and were highly purified (95-99%) by counterflow centrifugal centrifugation (15). Cell purity was checked by flow cytometry analysis using monoclonal antibodies to CD14 and CD11b (Becton Dickinson, San Jose, CA). Monocytes were cultured at 2 × 10^6 cells/ml for 24 h in 1 ml of RPMI 1640 medium (Whittaker, Walkersville, MD) supplemented with 10% fetal calf serum (Whittaker), 100 μg/ml penicillin, 100 μg/ml streptomycin and 0.03% glutamine and stimulated at the beginning of the culture with the following substances as indicated: heat-killed, formalin-fixed Staphylococcus aureus, Cowan I strain (SAC; Calbiochem, Cambridge, MA), LPS (Escherichia coli serotype 0127:B8; Sigma Chem. Co., St. Louis, MO) (16, 17), recombinant IFN-γ (Enzyme, Cambridge, MA), CD40L trimer (kindly provided by Immunex Corporation, Seattle, WA), IFN-α (Endogen, Cambridge, MA), or anti–IFN-γ (Endogen). One experiment was typically performed with the monocytes from one donor, and there was individual variability in IL-12 production (e.g., range for SAC plus IFN-γ stimulation 380-3,300 pg/ml IL-12 p70, mean ~1,750 pg/ml). Monocytes were incubated with heat-killed Histoplasma capsulatum (H.C.), (strain GS-57, kindly provided by Dr. R. Seder, Lymphokine Regulation Unit, LCI, NIAID, NIH) or iC3b-SRBC (2 × 10^7/ml) 2 h before stimulation as indicated. iC3b-SRBC were prepared

A abbreviations used in this paper: CMI, cell-mediated immunity; DTH, delayed type hypersensitivity; ECL, enhanced chemiluminescence; HC, Histoplasma capsulatum; HRP, horseradish peroxidase; ICAM-1, intercellular adhesion molecule 1; LPG, lipophosphoglycan; SAC, Staphylococcus aureus cells; STAT1, signal transducers and activators of transcription 1.
by sequential addition of anti-sheep erythrocyte antibodies (IgM; PharMingen, San Diego, CA) and C5-deficient human serum (Sigma) to SR BC as described (18, 19). The presence of IC3b on the cell surface was verified by flow cytometry showing a >100-fold increase in fluorescence intensity with a monoclonal antibody to the IC3b neoantigen (Quidel, San Diego, CA). Human PBM C were isolated by Ficoll-Hypaque density gradient centrifugation from leukocyte concentrates prepared by automated leukapheresis of healthy donors (total n = 5) as described (15). PBM C were cultured similar to monocytes, except that they were cultured at 1 × 10^6 cells/ml for 48 h and stimulated with PHA (Sigma). In some experiments, recombinant human IL-12, or recombinant IL-2 (PharMingen) were added as indicated.

Culture supernatants were assessed in duplicate by ELISA using kits or antibody pairs for IL-12 p70, TNF-α (both from R & D Systems), IL-12 p40; IL-10 (both from PharMingen), IL-6, IL-1β, IFN-α, IFN-γ (all from Endogen, Cambridge, MA), and TGFB1 (Genzyme). Unstimulated cultures (with or without added integrin antibodies) contained cytokines below the detection limit (IL-12 p70 > 5 pg/ml, IL-12 p40 > 20 pg/ml, IFN-α > 10 pg/ml, IL-10 > 100 pg/ml, TGFB1 > 50 pg/ml, IL-6 > 50 pg/ml; IL-1β > 25 pg/ml). For TNF-α, the detection limit was > 25 pg/ml and the background was below 20 pg/ml. Integrin antibodies were added at the beginning of the culture at the indicated concentrations and were obtained in a preservative-free preparation or dialyzed overnight before use. The antibodies and monocyte stimulants (e.g., CD40L trimer) contained low endotoxin levels as per information by the manufacturer and as demonstrated in appropriate assays performed in the presence of 1–5 μg/ml of polymyxin B (Sigma) which revealed little or no effect on IL-12 and TNF-α production. Antibodies were bound to plastic culture plates by overnight incubation at 10 μg/ml in 100 μl of carbonate buffer at 4°C followed by washing with PBS, and bound to polystyrene beads (goat anti–mouse IgG, Dynabeads M 450, Dynal, Lake Success, NY) according to the manufacturer’s instructions. L cells stably transfected with a plasmid expressing M450, Dynal, Lake Success, NY) according to the manufacturer’s instructions. L cells stably transfected with a plasmid expressing M450, Dynal, Lake Success, NY) according to the manufacturer’s instructions. L cells stably transfected with a plasmid expressing M450, Dynal, Lake Success, NY) according to the manufacturer’s instructions.
of other monocyte products such as TNF-α, IL-10, TGF-β, IL-6, and IL-1β (Fig. 1 C; Table 1) was not significantly altered by any of the above antibodies (as determined in the same cultures), nor was cell viability (trypan blue exclusion) or cell surface expression of CD14 (flow cytometry; data not shown). The one exception was an anti-CR3 3-induced reduction of IFN-α secretion (Table 1), a macrophage-derived cytokine previously shown to enhance IL-12-induced Th1 development (22). It is interesting to note that with higher doses of CD11b antibodies (25 μg/ml), we observed a further downregulation of IL-12 (<5 pg/ml) and a twofold increase in IL-6 (5,450 pg/ml), a cytokine dichotomy previously described in individuals with HIV infection (23).

We then studied the ability of natural CR3 ligands to suppress IL-12 production by using iC3b-coated sheep red blood cells (iC3b-SRBC) and heat-killed Histoplasma capsulatum (HC), an organism that binds to CR3 (3, 24). Similar to our findings with anti-CD11b, when human monocytes were incubated with iC3b-SRBC or heat-killed HC and subsequently stimulated with IFN-γ and either SAC, LPS, or CD40L trimer, we observed a downregulation of IL-12 p70 production (Figs. 3 and 4 A), whereas TNF-α and IL-10 levels were largely unaffected (Fig. 4 B). The inhibition of IL-12 production by HC was: (a) not due to direct suppression by IL-10, since the addition of anti-IL-10 antibodies (10 μg/ml) to the cultures only slightly increased
1990 CR3 and IL-12 Production

...the levels of IL-12; and (b) was related to the ability of HC to bind to β2 integrins, since the addition of a CD18 antibody (clone CLB LFA1/1 which did not directly inhibit IL-12 production, see Table 1) to these cultures partially reversed the suppressive effect of HC (threefold increase in IL-12 p70, i.e., to 40% of levels without HC).

Our findings with iC3b-SR BC expand upon a recent study by Karp et al. (25), who first demonstrated a role for complement regulatory proteins in the regulation of IL-12. It was shown that the in vitro infection of human monocytes with measles virus results in the suppression of IL-12 production and that such suppression was mediated through a measles virus receptor, CD46, which is an important complement regulatory protein that binds C3b (25). Thus, it appears that the binding of C3b to CD46, and the binding of iC3b to CR3, as may occur with the interaction of opsonized organisms with phagocytes, are two mechanisms by which complement components may suppress IL-12 production by human monocytes.

The suppression of IL-12 production by antibodies and ligands to CR3 could be due either to the transmission of a direct inhibitory signal through the CR3 molecule, or to...

**Figure 2.** Monocytic IL-12 production induced by various stimuli is suppressed by antibodies to CD11b. Secretion of IL-12 as determined by ELISA in cultures of human monocytes after stimulation with (A) LPS and IFN-γ, (B) SAC and IFN-γ, and (C) LPS alone (1 μg/ml), SAC alone (0.01%), SAC plus anti-IFN-γ (10 μg/ml), SAC plus IFN-α (10 ng/ml), and CD40L trimer (3 μg/ml) plus IFN-γ (1 μg/ml). Similar results were obtained with LPS plus increasing doses of IFN-γ, SAC plus increasing doses of IFN-γ, SAC plus TNF-α, TNF-β, and SAC plus anti-IL-10 (data not shown). One of three experiments is shown.

**Table 1.** Effects of Integrin Antibodies on the Secretion of a Variety of Cytokines by Highly Purified Human Monocytes

| Cytokine | None (clone 107.3) | IgG1 (G555-178) | CD11b (LM2/1) | CD18 (M1/70) | CD11a (M1/70) | CD11c (G43-25B) |
|----------|-------------------|----------------|--------------|--------------|---------------|----------------|
| IL-12 (p70) | 2,180 | 2,040 | 2,155 | 2,600 | 735 | 780 | 670 | 185 | 1,720 | 1,625 | 770 |
| IL-12 (p40) | 23,450 | 21,960 | 19,870 | 2,400 | 18,170 | 19,350 | 13,140 | 19,350 | 2,050 | 1,850 | 1,388 | 770 |
| IFN-α | 250 | 265 | ND | 30 | 18,170 | 13,140 | 19,350 | 13,140 | 19,350 | 1,850 | 1,388 | 770 |
| TNF-α | 1,850 | 2,133 | 2,325 | 2,575 | 2,100 | 2,638 | 2,050 | 1,850 | 3,188 | 2,025 | 3,150 | 2,800 |
| IL-10 | 5,200 | 5,750 | 5,700 | 4,550 | 5,500 | 4,300 | 5,300 | 5,500 | 5,175 | 2,950 | 2,650 | 2,800 |
| TGF-β | 2,350 | 2,675 | 2,375 | 3,300 | 2,650 | 2,275 | 2,625 | 3,175 | 2,950 | 2,650 | 2,800 |
| IL-6 | 2,650 | 3,050 | ND | 4,275 | 3,700 | 3,450 | 3,425 | 3,600 | 3,350 | 3,525 | 2,800 |
| IL-1β | 2,025 | 2,138 | ND | 2,050 | 2,052 | 2,050 | 2,363 | 1,875 | 2,338 | 2,020 | 1,875 | 2,020 |

Human monocytes (2 × 10^6/ml) were incubated with the indicated antibodies and stimulated with SAC (0.01%) plus IFN-γ (1 μg/ml). Cytokine levels from culture supernatants were determined as described in Materials and Methods. Data represent antibody concentration of 10 μg/ml. Data are means of duplicates from one experiment and are representative of three experiments. Percent suppression of IL-12 p70 in all three experiments was comparable, e.g., percent suppression of IL-12 p70 for clone LM2/1 ranged from 66-98%, for clone 44 (53-74%), for M1/70 (41-78%), for D12 (48-85%), for MEN-48 (82-99%), for CLB LFA1/1 (12-22%), for G43 25β (-16-33%), and for B-ly6 (37-79%). The baseline stimulated IL-12 p70 production varied in the three experiments (i.e., with cells from three different donors) from 1,060 to 2,180 pg/ml. ND denotes not determined.
the blocking of a positive signal for IL-12 production, as could be provided through interactions between CR3 and ICAM-1 on neighboring monocytes. To clarify this issue, we initially performed a number of studies which together suggested that important cell-cell interactions, especially via ICAM-1/CR3, are not particularly important for the induction of IL-12, and thus indirectly supported the conclusion that CR3 antibodies act via a direct inhibitory signal. First, we found that the relative production of IL-12 per monocyte after SAC and IFN-γ stimulation was not altered when cells were diluted over large ranges (≥1:1,000) before culture, i.e., resulting in decreased homotypic interactions (data not shown). Second, the addition of anti–ICAM-1 to monocyte cultures did not suppress IL-12 production (data not shown). And third, antibodies to CD11b (clone LM2/1) immobilized onto plastic culture plates, polystyrene beads coated with anti-mouse IgG (Dynal, Lake Success, NY), or to FcγRII receptor (CD32)-expressing L cells, completely inhibited SAC plus IFN-γ-induced IL-12 production (Fig. 5), suggesting that the membrane-fixed subset of CR3 (i.e., which remains unbound to the solid supports [26]) is not capable of providing a positive signal for IL-12 production through an interaction with ICAM-1 on neighboring monocytes.

Because these studies provided only indirect evidence that anti-CR3 was acting to transmit a negative signal to monocytes, we next looked more directly for intracellular targets of inhibitory signaling by CR3. Inhibition of tyrosine phosphorylation by CR3 signaling seemed possible since this effect had been previous demonstrated in a system that involves a CR3 ligand. Thus, it has been shown that tyrosine phosphorylation in response to IFN-γ was suppressed in macrophages infected with L. donovani [27], an organism binding to monocyte CR3 by either gp63 or lipophosphoglycan (LPG) [3, 7]. Furthermore, a similar phenomenon has been described in human monocytes after the binding of immune complexes [28], and a role for β1-integrin engagement in tyrosine dephosphorylation has been

**Figure 3.** Inhibition of monocyte IL-12 production by a natural ligand to CR3, iC3b-coated SRBC. iC3b-SRBC (2 × 10⁷/ml) were incubated with human monocytes (2 × 10⁶/ml) 2 h before stimulation with LPS (1 μg/ml) plus IFN-γ (1 μg/ml), SAC (0.01%) plus IFN-γ (1 μg/ml), or CD40L trimer (3 μg/ml) plus IFN-γ (1 μg/ml). IL-10 and TNF-α levels in the same culture supernatants were not significantly altered (data not shown). Data are representative of five experiments.

**Figure 4.** Secretion of IL-12 p70 by monocytes is suppressed by incubation with heat-killed Histoplasma capsulatum (H.C.). Heat-killed H.C. at the indicated concentrations was incubated with human monocytes 2 h before stimulation with LPS (1 μg/ml) plus IFN-γ (1 μg/ml), SAC (0.01%) plus IFN-γ (1 μg/ml), or CD40L trimer (3 μg/ml) plus IFN-γ (1 μg/ml) (A). (B) IL-10 and TNF-α levels from the same culture supernatants. Data are representative of five experiments, and percent suppression by H.C. was similar in all instances.
recently established (29). Thus, we explored the possibility that reduced production of IL-12 and IFN-α could follow a CR3-induced inhibition of IFN-γ signal transduction. As shown in Fig. 6, we found that incubation of monocytes with IFN-γ alone induced tyrosine phosphorylation of several proteins, one of which was identified as STAT1 (signal transducers and activators of transcription 1) by Western blotting with anti-STAT1 antibodies and by use of a positive tyrosine phosphorylated STAT1 control (Fig. 6). After preincubation with anti-CR3, IFN-γ-induced tyrosine phosphorylation of several proteins, including STAT1 was not seen, suggesting an inhibition of tyrosine phosphorylation, or alternatively, an accelerated dephosphorylation of tyrosine residues by an as yet unidentified protein tyrosine phosphatase.

The inhibition of IFN-γ-mediated STAT1 activation (i.e., phosphorylation) presents a potential mechanism by which signaling through CR3 could decrease monocyte IL-12 production, since there are two potential STAT1-binding sites (IFN-γ activation sequence [GAS] elements) in the human IL-12 p40 promoter (positions −127 to −119 and −277 to −268 of the published sequence), and since mutations with deletions at least one of these sites reduced transcription of a reporter gene (30). In addition, it is possible that the JAK-STAT-GAS signal transduction pathway may be important for the regulation of human IL-12 p35 gene transcription that is induced by stimulation with IFN-γ (30), or be involved in the expression of gene products that are indirectly responsible for the upregulation of IL-12 p35 or IL-12 p40 gene transcription or translation. Finally, the possibility that in addition to its effects on IFN-γ signaling, anti-CR3 suppressed IL-12 production through an IFN-γ-independent mechanism as well was supported by the observation that the low level of IL-12 induced by SAC alone, which was unchanged with the addition of anti-IFN-γ, was also inhibited with anti-CR3 (Fig. 2 C). Additional studies are needed to address these possibilities, as well as to address whether signaling via CR3 has effects on transcription factors other than STAT1, such as ets-protein family members, that have been more directly implicated in the regulation of IL-12 p40 in humans (30).

We finally extended our studies on functional effects of CR3 signaling to a murine model of IL-12–dependent septic shock. In this model, the intravenous injection of LPS results in symptoms of septic shock as well as high serum levels of IFN-γ and IL-12, all of which can be inhibited by the systemic administration of anti–IL-12 (31). We chose this in vivo model to study the effects of the antibodies to CR3 because, when compared to others, this model should be much less dependent on cell trafficking. This is because the injected LPS should be rapidly delivered to all lymphoid tissues and the response to LPS is unlikely to depend on significant migration of cells, at least within the first 3 or 6 h, the times at which we measured IL-12 and IFN-γ, respectively.

Thus, in our studies, BALB/c mice were given intraperitoneal injections of either CR3 antibodies (1 mg of clone M1/70 or 0.5 mg of clone 5C6 [8, 32]) or control rat IgG 1 h before LPS injection and were killed either 3 or 6 h later, at which time serum was obtained. As shown in Fig. 7, pretreatment with anti-CR3 (clone M1/70) reduced the serum levels of IL-12 (p40 and p70) by more than 4 and
2.5-fold, respectively, and similarly diminished the levels of IFN-γ 4-fold. The effects on IL-12 by treatment with M1/70 (or similar effects seen with clone 5C6; data not shown) were, consistent with prior studies of these antibodies (8, 13), not due to elimination of circulating leukocytes since total and differential white blood cell counts were similar in all treatment groups (data not shown). In addition, we found that whole spleen cells from anti-CR3–treated and LPS challenged mice stimulated in vitro with anti-CD3/anti-CD28 or PHA manifest reduced (two- to fourfold) IFN-γ production when compared to control mice (data not shown). In accordance with these findings, addition of anti-CD11b antibodies (10 μl/ml) markedly inhibited IFN-γ production in human monocyte cultures stimulated with either PHA (3,310 pg/ml IFN-γ for isotype control, 1,100 pg/ml for anti-CD11b [LM2/1]), plate-bound anti-CD3 plus anti-CD2 (3,250 pg/ml IFN-γ for isotype control, <10 pg/ml for anti-CD11b) or anti-CD2 plus anti-CD28 (2,450 pg/ml IFN-γ for isotype control, <10 pg/ml for anti-CD11b), but had no significant effect on cell viability or on cell proliferation (as determined by [3H]thymidine incorporation at 72 h). The above effects of anti-CR3 on IFN-γ production were reversed by addition of recombinant human IL-12 (1,100 pg/ml IFN-γ after PHA stimulation with anti-CR3, 3,210 pg/ml after addition of 20 ng/ml of IL-12) but not by IL-2 indicating the specificity for IL-12.

These data show that the IL-12 response, crucial for the initiation of most CMI functions (33), may be regulated by CR3 signaling. Thus, they provide insight into mechanisms underlying the ability of CR3 antibodies to abolish DTH reactions (8, 11, 12), to ameliorate Th1 cell–mediated autoimmune diseases including antigen-induced arthritis (34) and experimental allergic encephalomyelitis (35), and to enhance the severity of infections with L. monocytogenes (13) or T. gondii (14). In addition, our studies demonstrating the inhibition of IL-12 by HC and iC3b-SRBC suggest a mechanism for the impaired CMI accompanying infections with CR3 binding microorganisms (e.g., L. major, mycobacteria, HIV) (3, 36). In this regard they may provide a cogent reason for the diminished IL-12 production by mononuclear cells in HIV infected individuals (17) and in leishmaniasis (36). It is possible that CR3-induced suppression of IL-12 responses to the above microorganisms may result in suppressed monocyte nitric oxide production and respiratory burst, thereby explaining their ability to thrive in intracellular compartments.

Finally, our data, together with those recently developed by Karp et al. (25) showing that suppressed IL-12 in measles virus infection is mediated by the CD46 complement regulatory protein, establishes a new locus of control for T cell–mediated immunity via complement components. Thus, in disease states as well as in normal responses to invading microorganisms, signaling through CR3 may play an important role in regulating Th1–Th2 homeostasis. This is important for understanding the pathogenesis of infectious diseases, and may provide a new approach for therapeutic immunointervention targeting IL-12.

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**Figure 7.** Production of IL-12 and IFN-γ in a murine model of septic shock is suppressed by treatment with CR3 antibodies. Pretreatment with 1 mg of anti-CR3 (clone M1/70) markedly suppressed the serum levels of IL-12 p40 and p70 as well as serum levels of IFN-γ in BALB/c mice challenged by intravenous LPS (1 μg/mouse). Similar results were obtained with a different anti-CR3 antibody (clone 5C6). Data show the mean ± SD of groups consisting of three separately handled mice. *P < 0.001, **P < 0.0001, and ***P < 0.05 versus the control (i.e., rat Ig treated) group as determined by the Student’s test. Data are representative of five experiments.

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1993 Marth and Kelsall
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