

LUND UNIVERSITY

Critical role for complement receptor 3 (CD11b/CD18), but not for Fc receptors, in killing of Streptococcus pyogenes by neutrophils in human immune serum.

Nilsson, Maria E; Weineisen, Maria; Andersson, Tommy; Truedsson, Lennart; Sjöbring, Ulf

Published in: European Journal of Immunology

DOI: 10.1002/eji.200424850

2005

Link to publication

Citation for published version (APA):

Nilsson, M. E., Weineisen, M., Andérsson, T., Truedsson, L., & Sjöbring, U. (2005). Critical role for complement receptor 3 (CD11b/CD18), but not for Fc receptors, in killing of Streptococcus pyogenes by neutrophils in human immune serum. European Journal of Immunology, 35(5), 1472-1481. https://doi.org/10.1002/eji.200424850

Total number of authors: 5

General rights

Unless other specific re-use rights are stated the following general rights apply:

- Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the
- legal requirements associated with these rights

· Users may download and print one copy of any publication from the public portal for the purpose of private study or research.

- You may not further distribute the material or use it for any profit-making activity or commercial gain
 You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: https://creativecommons.org/licenses/

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117 221 00 Lund +46 46-222 00 00 The following pages constitute the final and revised manuscript of the article:

Critical role for complement receptor 3 (CD11b/CD18), but not for Fc receptors, in killing of *Streptococcus pyogenes* by neutrophils in immune human serum

Running title: Killing of S. pyogenes is mediated by CD11b/CD18

Maria Nilsson^[a], Maria Weineisen^{[a],[b]}, Tommy Andersson^[b], Lennart Truedsson^[a], and Ulf Sjöbring^[a]

Published in:

European Journal of Immunology Volume 35, Issue 5, Pages 1472 – 1481 Use of alternative location of the published version of the article rewuires journal subscription. Alternative location: http://dx.doi.org/10.1002/eji.200424850

From the Departments of Laboratory Medicine, ^[a]Section of Microbiology, Immunology and Glycobiology, Lund, and ^[b]Section of Experimental Pathology, Malmö University Hospital, Malmö, Lund University, Sweden

Keywords: complement receptor, Fc receptor, neutrophil, phagocytosis, Streptococcus

Address correspondence to: Maria Nilsson Department of Laboratory Medicine Lund University BMC B14, Tornavägen 10, 221 84 Lund, Sweden Phone: +46 46 222 85 92, Fax: +46 46 15 77 56 e-mail: maria.nilsson@mig.lu.se

Financial support: This work was supported by grants from the Swedish Research Council (grant no 9926), and the Swedish Foundation for Strategic Research,

Abbreviations: FcR – Fc receptor; mAb - monoclonal antibody; sCR1 – soluble complement receptor 1; cfu – colony forming units

The study was approved by the Ethical Council at Lund University and informed consent was obtained from all blood donors.

SUMMARY

During phagocytosis surface receptors on neutrophils interact with pathogens opsonized with complement factor C3b/iC3b and in some cases with antibodies. In immune human sera antibodies directed against surface-bound M proteins mediated killing of *Streptococcus pyogenes* by neutrophils. Surprisingly, blocking of the Fc receptors had little effect on the killing. In contrast, inhibition of C3b/iC3b generation, or blocking of the major neutrophil iC3b receptor CD11b/CD18, enabled *S. pyogenes* to grow efficiently in immune sera. Inhibition of CD11b/CD18, but not of CD32, the major neutrophil signalling Fc receptor, prevented streptococcus-induced NADPH oxidase-dependent respiratory burst, and blocking of C3b/iC3b formation inhibited streptococcus-induced activation of Cdc42, a small GTPase critically involved in transmitting proinflammatory signals to the cytoskeleton. Consequently, ligation of CD11b/CD18 by bacteria-bound iC3b is necessary for inducing a neutrophil response leading to elimination of *S. pyogenes* in immune human serum.

INTRODUCTION

Streptococcus pyogenes causes common mild infections such as tonsillitis and impetigo, but may also give rise to septicemia, necrotizing fasciitis, and toxic shock [1]. *S. pyogenes* grows in whole human blood from non-immune individuals as efficiently as it does in culture medium [2]. This is due to phagocytosis resistance primarily conferred by a class of surface proteins designated M proteins. Consequently, *S. pyogenes* lacking M protein are unable to grow in the system [3, 4]. Evidence suggests that the antiphagocytic property of the M protein is due to inhibition of complement deposition [5]. In blood containing M serotype specific antibodies (Abs) *S. pyogenes* are efficiently eliminated [5, 6]. The mechanisms whereby protective Abs contributes to the killing of streptococci are unknown.

In phagocytosis surface receptors on phagocytic cells interact with pathogens opsonized with complement factor C3b/iC3b, and in some cases with specifically bound IgG or IgA. These interactions trigger signals that lead to remodelling of the cytoskeleton and to subsequent engulfment of the pathogen [7]. Following uptake, the pathogens are killed through the combined action of oxidative and non-oxidative mediators [8, 9]. It is believed that the interaction between C3b or iC3b and complement receptors (CD35, CD11b/CD18 and CD11c/CD18) are sufficient for mediating uptake that is not associated with membrane protrusion or production of reactive oxygen species. In contrast, in phagocytosis mediated via Fc receptors (CD16, CD32, CD64 and CD89), phagocytosis is linked to pseudopod formation, induction of respiratory burst and expression of proinflammatory genes [10].

Much of the evidence for the different functional roles of the two phagocytic pathways emanate from *in vitro* experiments with purified reagents. We here show that the primary role of M protein-specific opsonizing Abs *ex vivo* is to promote complement deposition and activation on the bacterial surface, rather than to mediate FcR-mediated phagocytosis.

RESULTS

M protein-specific human antibodies mediate killing of S. pyogenes by neutrophils

To screen for donors of immune serum the ability of six *S. pyogenes* strains, representing different serotypes, to grow in whole blood from healthy individuals (donor I-III) was first analyzed (Fig. 1A). While, as previously noted [11], there was a considerable interexperimental variation, the relative outcome of the different experiments was consistent: M3 streptococci was always killed in blood from donor I, M1 and M3 streptococci were both killed in blood from donor II, whereas M1 and M5 bacteria were killed in blood from donor III (Fig. 1A). The blood from a fourth donor supported growth of all of these strains (data not shown). Similar results were obtained when substituting blood with purified neutrophils and serum (data not shown). Experiments were M3 streptococci, whereby blood cells and plasma from donor I were separated and then mixed crosswise with cells or plasma from a non-immune donor, showed that the specificity of the killing depended on plasma component(s) rather than on cellular factors (Fig. 1B). The component was identified as IgG since addition of this protein, purified from donors I and II, to blood from non-immune donors supported killing of M3 and M1 bacteria, respectively (Fig. 1C).

To study the specificity of these Abs, M1 or M3 streptococci were grown in a mixture of neutrophils and immune serum supplemented with soluble rM1 or rM3: The M3 strain demonstrated significant growth in serum I supplemented with rM3 as compared to serum I supplemented with rM1 (Fig. 2A). Conversely, addition of rM1, but not of rM3, supported growth of M1 streptococci in serum from donor II (Fig. 2A). These data suggest that the homologous M protein consumed the opsonizing Abs in a serotype-restricted manner. We then studied if IgG in opsonizing (from donor I) and non-opsonizing sera displayed different reactivity with the entire M3 protein, which contains epitopes common to many M proteins, or with a recombinant fragment containing only the unique N-terminus (rM3N₅₈). Alhough there was a difference in the anti-M3 titer between the two sera (Fig. 2B), the difference was much greater for the Abs directed against the M3N₅₈ fragment (Fig. 2C). Thus, opsonizing Abs are primarily directed against the variable N-terminal region of M proteins (Fig. 2D).

CD11b/CD18 mediates killing of S. pyogenes by neutrophils in immune serum

Antibodies could serve as opsonins for phagocytosis, act as enhancers of complement activation, or both. To distinguish between these alternatives, a panel of function-blocking mAbs directed against relevant complement and FcRs, were used to inhibit neutrophil killing of the M1 and M3 strains in immune serum, at concentrations previously described to block receptor-binding (Table 1). For the

most critical mAbs, their function-blocking ability was confirmed by flow cytometry experiments (Table 1).

CD11b/CD18 is the major receptor for iC3b on neutrophils. For both M1 and the M3, addition of a mAb against CD11b, known to block iC3b-binding, or of F(ab')₂ fragments of this mAb, resulted in streptococcal growth. The finding was confirmed using a second blocking anti-CD11b mAb. Isotype control mAbs did not influence bacterial growth. Blocking of the second iC3b-receptor CD11c/CD18, that is expressed in relatively low levels on neutrophils, resulted in a small but significant effect in the M3-donor I system. Inhibition of CD35, that interacts with C3b, failed to influence bacterial killing. Finally, blocking of CD11a/CD18, that has no complement-binding, also lacked effect.

CD32 and CD16 are highly expressed in neutrophils. While binding to CD32 directly activates the cytoskeleton and respiratory burst [10], CD16 requires co-stimulation via CD11b/CD18 to induce these processes [20]. Ligation of CD89, the neutrophil FcR for IgA, can mediate respiratory burst [21]. Surprisingly, blocking of the major signalling receptor CD32 with two different mAbs failed to influence streptococcal growth (Table 1). Inhibition of CD16 and CD89 resulted in a small but significant effect in the M3 - donor I system but lacked effect in the M1 - donor II system. Simultaneous addition of all three FcR mAbs produced no additional inhibition (data not shown).

Neutrophil killing of *S. pyogenes* in immune serum is mediated through antibody-dependent complement activation

Apart from iC3b, CD11b/CD18 interacts with several serum proteins. To establish the role of complement activation vs. other plausible mechanisms, we blocked C3b generation in the bactericidal assay at two different levels: Compstatin specifically binds to C3 and blocks its cleavage [22], while sCR1 competitively binds C3b and inhibits the C3 convertase [23]. Both inhibitors reduced killing of M1 and M3 bacteria in the corresponding immune sera although compstatin was less efficient in the M1-donor II system (Fig. 3A).

We then studied the relative role of the classical and the alternative pathways for streptococcal opsonization. Analysis of covalently bound complement components showed that surface-deposition of both C3b and C4b was more pronounced following incubation in immune serum than in non-immune serum (Fig. 3B), primarily suggesting a role for the classical pathway. Experiments with C1q- and factor D-depleted sera suggested that the alternative pathway plays a critical role, since killing of the bacteria was more efficiently restored by supplementation with factor D than

with C1q (Fig. 3C). Moreover, a mAb against properdin more efficiently promoted growth of M3 bacteria in donor I serum than did a blocking anti-C1s mAb (Fig. 3D). Together, these findings suggest that while the classical pathway is important, the alternative pathway also significantly contributes to the killing of *S. pyogenes* in immune serum.

Streptococcus-induced respiratory burst by neutrophils in immune serum depends on iC3b interactions with CD11b/CD18

Respiratory burst is an important feature of neutrophil activation. In general, activation of the NADPH oxidase system is believed to require stimulation of FcRs, and in particular of CD32 [10, 24], although if immobilized on surfaces or fed with particles that cannot be ingested, respiratory burst in neutrophils can be induced through CD11b/CD18 [25]. Consistent with the observation that killing of M3 streptococci was dependent on iC3b-CD11b/CD18 binding, we found that respiratory burst induced by the streptococci in immune serum was reduced by sCR1 and anti-CD11b, but not by anti-CD32 (Fig. 4). Thus, interactions between streptococcus-bound iC3b and CD11b/CD18 is necessary for activation of the NADPH system in neutrophils. Whether respiratory burst directly contributes to killing of *S. pyogenes* remains to be established.

Inhibition of C3b/iC3b formation on streptococci reduces neutrophil levels of activated Rac2 and Cdc42

The Rho family GTPases Cdc42 and Rac control membrane ruffling (Cdc42), transcriptional activation of proinflammatory genes (Cdc42) and respiratory burst (Rac). We followed the content of the activated forms of Cdc42 and Rac2 (that represents >90% of Rac in neutrophils) in neutrophils following contact with M3 streptococci in immune serum, using a GST-PAKcrib fusion protein that only binds GTP-bound Cdc42 and Rac [26]. There was a significant increase of activated Cdc42 in neutrophils after incubation with bacteria in immune serum (Fig. 5B). This fits with observations by Dib *et al.* [27], who showed that engagement of β_2 integrins induced an increase of GTP-bound Cdc42. Moreover, inhibition of complement by sCR1 reduced activation of neutrophil Cdc42 in the immune system (Fig. 5C).

The levels of activated Rac2 in neutrophils were not increased by M3 bacteria (Fig. 5A). However, Dib *et al.* demonstrated that although engagement of β_2 integrins resulted in a decrease of the GTP-bound form of Rac2, it caused translocation of Rac2 to the plasma membrane [27], where it can complex with p47^{*phox*} and p67^{*phox*} to form the active NADPH oxidase [28]. Therefore, an increase of the total amount of GTP-bound Rac2, may not be necessary for induction of respiratory burst.

Together these data suggest that these Rho GTPases are involved in mediating signals induced by iC3b – complement receptor interactions *ex vivo*.

DISCUSSION

The present study clearly demonstrates that CD11b/CD18 plays a pivotal role for neutrophilmediated killing of *S. pyogenes* in human immune serum. Furthermore, experiments with complement inhibitors suggest that the most important opsonizing factor in this system is bacteriabound iC3b, a major ligand for this integrin. Since experiments with FcR-blocking Abs surprisingly showed a limited impact, the most important role of the M protein-specific Abs is to mediate neutrophil killing by initiating complement activation and formation of bacteria-bound iC3b.

It is assumed that signalling through FcRs plays a critical role for the ability of phagocytes to dispose of microorganisms. Thus, ligation of FcyRIIA, but not of CD11b/CD18, leads to membrane ruffling, transcriptional activation of proinflammatory genes, and activation of the NADPH oxidase, through the small GTPases Rac and Cdc42 [10]. The limited effect by FcR-inhibition on streptococcal killing was therefore unexpected. The possibility remains that relatively few FcRs need to be activated to provide the required signals when β_2 -integrins are stimulated simultaneously. However, the demonstration that streptococcus-induced respiratory burst could be effectively blocked by anti-CD11b mAbs supports the concept that CD11b/CD18, at least under certain conditions, can transmit signals resulting in activation of the NADPH oxidase. In this context it should be pointed out that the assumption that FcR ligation plays the critical role for induction of oxidative burst largely emanates from experiments in highly defined systems using inert particles and defined reagents. These conditions do not readily translate to the ex vivo conditions used here, where simultaneous signalling through other receptors could make the requirement for FcR-stimulation redundant. Thus, immobilization of the neutrophils on matrix proteins can induce oxidative burst in neutrophils, particularly in the presence TNFa [29, 30]. Clearly, in the bactericidal assay with growing bacteria there are a number of ways through which such co-stimulatory pathways can be induced.

The experiments with complement-depleted sera and Abs capable of blocking complement provided surprising results. Thus, blocking of the alternative pathway was efficient in augmenting killing as blocking of the classical pathway, and factor D, but not C1q, could reconstitute the killing potential of C1q/factor D depleted immune serum. The alternative pathway could be activated through amplification of the classical pathway or through direct activation by the protective Abs [31, 32]. A direct activation by the Abs is not unlikely since it is known that both the Fc and Fab fragments of human IgG contain regions that are vulnerable to nucleophilic attack by C3b [32, 33].

The finding that the primary role of human M specific Abs is to increase streptococcus-associated C3b/C4b generation lends to speculation on the molecular basis for the antiphagocytic function of M proteins and in particular of their N-terminal variable region. Some evidence suggest that one role of the N-terminal region may be to acquire highly specific functions that down-regulate opsonization, including the binding of complement regulatory proteins [34, 35]. However, many M proteins, including M3, do not bind soluble complement regulators [36]. Therefore, other and less specific mechanisms may be just as important. For example, the overall negative charge of M proteins could function to repel interactions with neutrophils that also have an overall negative surface charge [37]. In analogy with this hypothesis, it is possible that the amino acid composition and/or distribution in the N-terminal region of M proteins has evolved not to provide a site for nucleophilic attack by C3b. In this scenario the role of the protective antibodies, in addition to activating the classical pathway, could be to affect the charge in order to facilitate C3b binding directly to the N-terminal region. Alternatively, protective antibodies could provide a scaffold upon which iC3b can be deposited. Both of these mechanisms are compatible with a direct role for the alternative pathway in antibody-dependent killing of *S. pyogenes*.

MATERIALS AND METHODS

Bacterial strains

The strains 90-226 (M1), 950771 (M3), Manfredo (M5), BA414 (M12), IRP168 (M18), and D943 (M22) are wild-type *S. pyogenes* isolates.

Blood, serum and neutrophils

Blood, serum and neutrophils were donated by healthy volunteers. Fresh heparinized blood was used to screen for donors of opsonizing serum. Serum was used immediately or stored at -80°C until use. Neutrophils were isolated from fresh heparinized blood as described [27]. Neutrophils were resuspended in RPMI 1640 with glutamax.

Opsonizing human antibodies and receptor-blocking monoclonal antibodies

Opsonizing antibodies were purified from immune serum of healthy individuals on a protein G coupled Hi-trap column. The set of receptor-blocking mouse mAbs used are described in Table 1. $F(ab')_2$ fragments were made using the Immunopure $F(ab')_2$ preparation kit (Pierce).

Bactericidal test

Todd-Hewitt broth was inoculated with 0.25 ml of an overnight culture, and the streptococci were grown to log phase ($OD_{620} = 0.150$). Bacteria (1 x 10^3 /ml) were incubated either with 0.25 ml of human blood or with a mixture of neutrophils (1 x 10^6 /ml), 20 % serum and RPMI 1640 in a total volume of 0.30 ml. Incubation was done in 5% CO₂ at 37°C for 3 h, with end-over-end rotation. Samples were withdrawn after 0 and 3 h and plated on blood agar. The multiplication factor was calculated as the number of cfu after 3 h divided by the number of cfu in the beginning of the experiment. There is an inter-experimental variation due to variable streptococcal chain length, variation of neutrophil activation and a time-dependent decline (over months) of the killing ability of the sera. The growth rates in different non-immune sera also vary. The long time of incubation further amplifies variation introduced by any of these factors.

Recombinant M proteins and solid phase radioimmunoassay

Cloning and expression of rM1 has been described [38]. The *emm3* gene and a fragment encoding the N-terminal 58 residues of the M3 protein (rM3N₅₈) were amplified by PCR and cloned into the expression plasmids pET25b (Novagen) for rM3 and pGEX-5x-3 for rM3N₅₈. Recombinant proteins were purified from *E. coli* lysates on fibrinogen-Sepharose 4B for rM1 and rM3 and on glutathione-Sepharose 4B for rM3N₅₈. For detection of rM3-specific human Abs, microtiter plates were coated with rM3 or rM3N₅₈ (0.5 μ g/well) by 6 h incubation at 4°C. The wells were blocked

with phosphate-buffered saline (PBS; 131 mM NaCl, 10 mM Na₂HPO₄·2H₂O, 0.4 mM KH₂PO₄, pH 7.2), containing 0.25% Tween and 0.25% gelatine. Serum was diluted in PBS in five-fold dilutions, added to the wells and incubated for 3 h at 20°C with shaking. Bound Abs were detected with [¹²⁵I]-protein G in a γ -counter.

Flow cytometry

To measure respiratory burst, $10x10^6$ streptococci, that had been grown to log phase (OD₆₂₀ = 0.4), were mixed with 5 x 10^5 neutrophils, that had been incubated with with 50 mM dihydrorhodamine-123 at 37°C for 15 min, 20% serum, RPMI, and Abs (30 µg/ml), or sCR1 (100 µg/ml), in a final volume of 0.50 ml and incubated at 37°C for 30 min with end-over-end rotation. The samples were washed twice and then kept on ice until analyzed by flow cytometry. To assess the inhibitory capacity of FcR-blocking mAbs, *S. cerviciae* particles were opsonized with Alexa Flour 488 conjugated rabbit anti-yeast Abs for 15 min at 37°C. The particles were then incubated with neutrophils that had been preincubated with blocking or isotype control mAbs, for 15 min at 37°C. For the anti-CD11b mAb, Alexa Flour 488 labelled *S. cerviciae* particles were opsonized with neutrophils that had been preincubated with blocking or isotype control mAbs, for 15 min at 37°C. The particles were incubated with neutrophils that had been preincubated with blocking or isotype control mAbs, for 15 min at 37°C. For the anti-CD11b mAb, Alexa Flour 488 labelled *S. cerviciae* particles were incubated in RPMI medium 45 min, 37°C, washed twice and then analyzed by flow cytometry.

Hemolytic assay

Complement function was measured in hemolysis assays as described [39]. For the classical pathway, sheep erythrocytes, treated with rabbit IgM to yield optimally sensitized cells, were mixed with serum in veronal buffered saline (VBS; 1.28 mM sodium barbiturate, 4.5 mM barbiturate, 0.14 M sodium chloride, pH 7.4) containing 0.15 mM Ca²⁺ and 0.5 mM Mg²⁺. Alternative pathway-mediated hemolysis was determined by mixing rabbit erytrocytes, serum, and VBS containing 10 mM EGTA and 2.5 mM MgCl₂. All samples were incubated for 20 min 37°C, then put on ice and VBS containing 10 mM EDTA was added to stop the reaction. For total hemolysis 0.1% NaHCO₃ was added. The erythrocytes were pelleted and the lysis was quantified by measuring the OD₅₄₁ of the supernatants, expressing the complement activity as per cent of total hemolysis.

Complement related reagents

The anti-properdin mAb blocked 84% of the alternative pathway. The anti-C1s mouse mAb M241 blocked 28% of the classical pathway in the hemolytic assays. The complement inhibitors compstatin [22] and recombinant sCR1 [40] were used at 70 and 100 μ g/ml, respectively. At these concentrations compstatin blocked 96% and 67% of the alternative and classical pathway,

respectively, while sCR1 blocked >90% of the activity of both pathways. The generation of serum selectively depleted of C1q and factor D has been described previously [41]. Briefly, serum was supplemented with 5 mM EDTA and dialysed against 50 mM phosphate buffer pH 7.3 with 2 mM EDTA and sodium chloride was added to yield a final conductance of 13 mS/cm². The dialysed serum was applied to a Biorex 70 column. Material not bound to the resin was collected and dialysed against VBS containing 0.15 mM Ca²⁺ and 0.5 mM Mg²⁺. The material was then concentrated. Reconstitution with C1q restored 100% of the classical pathway and 0% of the alternative pathway, while and factor D restored 96% of the alternative pathway and 30% of the classical pathway. C1q and factor D were kind gifts from Dr. A. Sjöholm, Lund University, Sweden.

Analysis of C3 and C4 fragments covalently bound to streptococci

C3/C4 fragments were released from bacteria as described [42]. Briefly bacteria (5 x 10⁸ cfu) were incubated in 1.0 ml 50% serum in PBS for 30 min and subsequently washed twice in PBS supplemented with 1% SDS. Pellets were then resuspended in 0.25 ml 1 M hydroxylamine pH 9.0 and 1% SDS and incubated for 60 min at 37°C. Bacteria were pelleted and the supernatant was reduced with 1% SDS and 10 mM dithiothreitol for 60 min at 37°C and then alkylated with 22 mM iodoacetamide in 10 mM Tris and 1 mM EDTA, pH 8.0 for 60 min at 37°C. The eluates were analyzed by SDS-PAGE and immunoblotting. Released fragments were detected using polyclonal anti-C3 (Sigma) or anti-C4 Abs followed by [¹²⁵I]-protein G and then visualized by autoradiography.

Activation of Cdc42 and Rac2

Neutrophils (5 X 10^6 /ml) were incubated with bacteria (1.5 X 10^7 /ml) in RPMI 1640 with 20% serum and 10% Todd-Hewitt broth at 37°C for 0, 15 or 30 min. The neutrophils were then lysed as described [27]. A GST-fusion protein expressing the Rac/Cdc42 binding domain of PAK1B [43] was added and the samples were incubated on ice for 5 min and were then centrifuged (14 000 x g, 10 min, 4°C). The cleared lysates were transferred to new tubes containing glutathione-Sepharose 4B beads (40 µl) and incubated for 60 min at 4°C. Following three washes with ice-cold washing buffer (25 mM Tris-HCl pH 7.5, 30 mM MgCl₂, 500 mM NaCl, 1% Triton X-100, 1 mM DTT, 2 mM Na₃VO₄) bound proteins were eluted by boiling the beads in 40 µl of sample buffer for 10 min. The proteins were separated by 12% SDS-PAGE and transferred to PVDF membranes. After blocking and washing steps, the membranes incubated with an anti-Cdc42 mAb followed by horseradish peroxidase-conjugated anti-mouse IgG and visualized by enhanced chemiluminescence. The membranes were then stripped and subsequently blotted for Rac2 (rabbit IgG C-11).

ACKNOWLEDGEMENTS

We sincerely thank Dr. Nancy Hogg, Leukocyte Adhesion Laboratory, ICRF, London, UK, Dr. Eric. J. Brown, UCSF, San Francisco, Dr. M. J. Glennie, University of Southampton, UK, and Dr. M. Matsumoto, Osaka University Graduate School of Medicine, Osaka, Japan for generously providing monoclonal antibodies. We are also very grateful to Dr. John D. Lambris, Department of Pathology and Laboratory Medicine, University of Pennsylvania for compstatin, Dr. Carolyn Pettey, Avant Immunotherapeutics Inc., Needham, MA for sCR1, Dr. Pontus Aspenström Ludwig Institute for Cancer Research, Uppsala, Sweden for GST-PAKcrib and Dr Anders Sjöholm, Institute for Laboratory Medicine, Lund University, Sweden for factor D and C1q. Malgorzata Berlikowski and Birgitta Gullstrand are gratefully acknowledged for expert technical assistance.

REFERENCES

- 1 **Bisno, A. L. and Stevens, D. L.,** Streptococcal infections of skin and soft tissues. *N Engl J Med* 1996. **334**: 240-245.
- Lancefield, R. C., Current knowledge of type-specific M antigens of group A streptococci. *J. Immunol.* 1962.
 89: 307-313.
- 3 **Thern, A., Wästfelt, M. and Lindahl, G.,** Expression of two different antiphagocytic M proteins by Streptococcus pyogenes of the OF+ lineage. *J Immunol* 1998. **160**: 860-869.
- 4 **Podbielski, A., Schnitzler, N., Beyhs, P. and Boyle, M. D.,** M-related protein (Mrp) contributes to group A streptococcal resistance to phagocytosis by human granulocytes. *Mol Microbiol* 1996. **19**: 429-441.
- 5 **Jacks-Weis, J., Kim, Y. and Cleary, P. P.,** Restricted deposition of C3 on M+ group A streptococci: correlation with resistance to phagocytosis. *J Immunol* 1982. **128**: 1897-1902.
- 6 **Lancefield, R. C.,** Persistence of type specific antibodies in man following infection with group A streptococci. *J. Exp. Med.* 1959. **110**: 271.
- 7 Vidarsson, G., van de Winkel, J.G.J., Fc receptor and complement receptor-mediated phagocytosis in host defence. *Curr Opin Infect Dis* 1998. **11**: 271-278.
- 8 **Reeves, E. P., Lu, H., Jacobs, H. L., Messina, C. G., Bolsover, S., Gabella, G., Potma, E. O., Warley, A., Roes, J. and Segal, A. W.,** Killing activity of neutrophils is mediated through activation of proteases by K+ flux. *Nature* 2002. **416**: 291-297.
- 9 **Hampton, M. B., Kettle, A. J. and Winterbourn, C. C.,** Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. *Blood* 1998. **92**: 3007-3017.
- 10 **Caron, E. and Hall, A.,** Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases. *Science* 1998. **282**: 1717-1721.
- 11 **Kotarsky, H., Gustafsson, M., Svensson, H. G., Zipfel, P. F., Truedsson, L. and Sjöbring, U.,** Group A streptococcal phagocytosis resistance is independent of complement factor H and factor H-like protein 1 binding. *Mol Microbiol* 2001. **41**: 817-826.
- 12 **Sanchez-Madrid, F., Nagy, J. A., Robbins, E., Simon, P. and Springer, T. A.,** A human leukocyte differentiation antigen family with distinct alpha-subunits and a common beta-subunit: the lymphocyte function-associated antigen (LFA-1), the C3bi complement receptor (OKM1/Mac-1), and the p150,95 molecule. *J Exp Med* 1983. **158**: 1785-1803.
- 13 **Dana, N., Todd, R. F., 3rd, Colten, H. R. and Arnaout, M. A.**, A dysfunctional Mo1 glycoprotein is present on a subline of the KG1 acute myelogenous leukemia cell line. *J Immunol* 1987. **138**: 3549-3554.
- 14 **Malhotra, V., Hogg, N. and Sim, R. B.,** Ligand binding by the p150,95 antigen of U937 monocytic cells: properties in common with complement receptor type 3 (CR3). *Eur J Immunol* 1986. **16**: 1117-1123.
- 15 Perussia, B., Trinchieri, G., Jackson, A., Warner, N. L., Faust, J., Rumpold, H., Kraft, D. and Lanier, L. L., The Fc receptor for IgG on human natural killer cells: phenotypic, functional, and comparative studies with monoclonal antibodies. *J Immunol* 1984. 133: 180-189.
- 16 **Looney, R. J., Abraham, G. N. and Anderson, C. L.,** Human monocytes and U937 cells bear two distinct Fc receptors for IgG. *J Immunol* 1986. **136**: 1641-1647.
- 17 **Greenman, J., Tutt, A. L., George, A. J., Pulford, K. A., Stevenson, G. T. and Glennie, M. J.,** Characterization of a new monoclonal anti-Fc gamma RII antibody, AT10, and its incorporation into a bispecific F(ab')2 derivative for recruitment of cytotoxic effectors. *Mol Immunol* 1991. **28**: 1243-1254.
- 18 O'Shea, J. J., Brown, E. J., Seligmann, B. E., Metcalf, J. A., Frank, M. M. and Gallin, J. I., Evidence for distinct intracellular pools of receptors for C3b and C3bi in human neutrophils. *J Immunol* 1985. 134: 2580-2587.
- 19 **Zhang, W., Bi, B., Oldroyd, R. G. and Lachmann, P. J.**, Neutrophil lactoferrin release induced by IgA immune complexes differed from that induced by cross-linking of fcalpha receptors (FcalphaR) with a monoclonal antibody, MIP8a. *Clin Exp Immunol* 2000. **121**: 106-111.
- 20 **Zhou, M. J. and Brown, E. J.,** CR3 (Mac-1, alpha M beta 2, CD11b/CD18) and Fc gamma RIII cooperate in generation of a neutrophil respiratory burst: requirement for Fc gamma RIII and tyrosine phosphorylation. *J Cell Biol* 1994. **125**: 1407-1416.
- 21 **Shen, L.,** A monoclonal antibody specific for immunoglobulin A receptor triggers polymorphonuclear neutrophil superoxide release. *J Leukoc Biol* 1992. **51**: 373-378.
- 22 Sahu, A., Kay, B. K. and Lambris, J. D., Inhibition of human complement by a C3-binding peptide isolated from a phage-displayed random peptide library. *J Immunol* 1996. **157**: 884-891.
- 23 Medof, M. E., Iida, K., Mold, C. and Nussenzweig, V., Unique role of the complement receptor CR1 in the degradation of C3b associated with immune complexes. *J Exp Med* 1982. **156**: 1739-1754.
- 24 **Kobayashi, S. D., Voyich, J. M., Buhl, C. L., Stahl, R. M. and DeLeo, F. R.,** Global changes in gene expression by human polymorphonuclear leukocytes during receptor-mediated phagocytosis: cell fate is regulated at the level of gene expression. *Proc Natl Acad Sci U S A* 2002. **99**: 6901-6906.
- 25 Serrander, L., Larsson, J., Lundqvist, H., Lindmark, M., Fällman, M., Dahlgren, C. and Stendahl, O., Particles binding beta(2)-integrins mediate intracellular production of oxidative metabolites in human neutrophils independently of phagocytosis. *Biochim Biophys Acta* 1999. **1452**: 133-144.

- 26 **Benard, V., Bohl, B. P. and Bokoch, G. M.,** Characterization of rac and cdc42 activation in chemoattractantstimulated human neutrophils using a novel assay for active GTPases. *J Biol Chem* 1999. **274**: 13198-13204.
- 27 Dib, K., Melander, F., Axelsson, L., Dagher, M. C., Aspenström, P. and Andersson, T., Down-regulation of Rac Activity during {beta}2 Integrin-mediated Adhesion of Human Neutrophils. *J Biol Chem* 2003. 278: 24181-24188.
- 28 Benard, V., Bokoch, G. M. and Diebold, B. A., Potential drug targets: small GTPases that regulate leukocyte function. *Trends Pharmacol Sci* 1999. **20**: 365-370.
- 29 Nathan, C., Srimal, S., Farber, C., Sanchez, E., Kabbash, L., Asch, A., Gailit, J. and Wright, S. D., Cytokine-induced respiratory burst of human neutrophils: dependence on extracellular matrix proteins and CD11/CD18 integrins. *J Cell Biol* 1989. **109**: 1341-1349.
- 30 **Bansal, V. S., Vaidya, S., Somers, E. P., Kanuga, M., Shevell, D., Weikel, R. and Detmers, P. A.,** Small molecule antagonists of complement receptor type 3 block adhesion and adhesion-dependent oxidative burst in human polymorphonuclear leukocytes. *J Pharmacol Exp Ther* 2003. **304**: 1016-1024.
- 31 Mak, L. W., Lachmann, P. J. and Majewski, J., The activation of the C3b feedback cycle with human complement components. I. Through the classical pathway. *Clin Exp Immunol* 1977. **30**: 200-210.
- 32 **Ratnoff, W. D., Fearon, D. T. and Austen, K. F.,** The role of antibody in the activation of the alternative complement pathway. *Springer Semin Immunopathol* 1983. **6**: 361-371.
- 33 Selander, B., Kayhty, H., Wedege, E., Holmström, E., Truedsson, L., Söderström, C. and Sjöholm, A. G., Vaccination responses to capsular polysaccharides of Neisseria meningitidis and Haemophilus influenzae type b in two C2-deficient sisters: alternative pathway-mediated bacterial killing and evidence for a novel type of blocking IgG. *J Clin Immunol* 2000. **20**: 138-149.
- 34 **Thern, A., Stenberg, L., Dahlbäck, B. and Lindahl, G.,** Ig-binding surface proteins of Streptococcus pyogenes also bind human C4b-binding protein (C4BP), a regulatory component of the complement system. *J Immunol* 1995. **154**: 375-386.
- 35 **Horstmann, R. D., Sievertsen, H. J., Knobloch, J. and Fischetti, V. A.,** Antiphagocytic activity of streptococcal M protein: selective binding of complement control protein factor H. *Proc Natl Acad Sci U S A* 1988. **85**: 1657-1661.
- 36 Perez-Caballero, D., Alberti, S., Vivanco, F., Sanchez-Corral, P. and Rodriguez de Cordoba, S.,
 Assessment of the interaction of human complement regulatory proteins with group A Streptococcus.
 Identification of a high-affinity group A Streptococcus binding site in FHL-1. *Eur J Immunol* 2000. 30: 1243-1253.
- **Fischetti, V. A.,** Requirements for the opsonic activity of human IgG directed to type 6 group A streptococci: net basic charge and intact Fc region. *J Immunol* 1983. **130**: 896-902.
- 38 Åkesson, P., Schmidt, K. H., Cooney, J. and Björck, L., M1 protein and protein H: IgGFc- and albuminbinding streptococcal surface proteins encoded by adjacent genes. *Biochem J* 1994. **300**: 877-886.
- 39 Nilsson, U. R. and Nilsson, B., Simplified assays of hemolytic activity of the classical and alternative complement pathways. *J Immunol Methods* 1984. **72**: 49-59.
- 40 Weisman, H. F., Bartow, T., Leppo, M. K., Marsh, H. C., Jr., Carson, G. R., Concino, M. F., Boyle, M. P., Roux, K. H., Weisfeldt, M. L. and Fearon, D. T., Soluble human complement receptor type 1: in vivo inhibitor of complement suppressing post-ischemic myocardial inflammation and necrosis. *Science* 1990. **249**: 146-151.
- 41 **Sjöholm, A. G., Selander, B., Östenson, S., Holmström, E. and Söderström, C.,** Normal human serum depleted of C1q, factor D and properdin: its use in studies of complement activation. *Apmis* 1991. **99**: 1120-1128.
- 42 **Gordon, D. L., Rice, J., Finlay-Jones, J. J., McDonald, P. J. and Hostetter, M. K.,** Analysis of C3 deposition and degradation on bacterial surfaces after opsonization. *J Infect Dis* 1988. **157**: 697-704.
- Edlund, S., Landström, M., Heldin, C. H. and Aspenström, P., Transforming growth factor-beta-induced mobilization of actin cytoskeleton requires signaling by small GTPases Cdc42 and RhoA. *Mol Biol Cell* 2002. 13: 902-914.

LEGENDS

Figure 1. Antibodies mediate strain-specific killing of S. pyogenes in human blood

A. The growth of *S. pyogenes* expressing the indicated M proteins was measured in blood from three donors. **B.** The cells and plasma in blood from immune (I) and non-immune (N) donors were separated by centrifugation and recombined, as indicated. The ability of M3 streptococci to grow in the different cell/plasma mixtures was then measured. **C**. The ability of M1 and M3 streptococci to grow in blood from non-immune donors (N) that had been supplemented with buffer or with IgG purified from donor I or II was measured. Multiplication factors were calculated as the ratio between the number of cfu after 3 h and at the beginning of the experiment. The data represent means and range of 3–5 independent experiments. The statistical significance was assessed by the paired Student's *t*-test; as indicated * P < 0.05, ** P < 0.01.

Figure 2. Human protective antibodies are directed against the N-terminal region of the M3 protein

A. The ability of M1 and M3 streptococci to multiply in mixtures of neutrophils and serum was monitored as in Fig. 1. M1 streptococci were mixed with serum from donor II, while M3 bacteria were mixed with serum from donor I. Neutrophils and M proteins (rM1 and rM3) (100 μ g/ml), or buffer (PBS), were added as indicated. The growth of M1 and M3 bacteria in non-immune serum was also measured (N, dotted bars). **B** and **C**. rM3 (B) or rM3N₅₈ (C) were immobilized in microtiter plates and were then probed with immune (donor I) or non-immune serum. Bound IgG was then detected with [¹²⁵I]-protein G. The data represent means and the range of 3–5 independent experiments. The statistical significance was assessed by the paired Student's *t*-test; as indicated * P < 0.05, ** P < 0.01, *** P < 0.001. **D**. Schematic model of a typical M protein, indicating the localization of the opsonizing epitope(s). The hatched section indicates the localization of rM3N₅₈ in the M3 protein.

Figure 3. Important role of the alternative pathway for the killing of *S. pyogenes* in immune human serum

A. Multiplication of M1 and M3 streptococci in mixtures of neutrophils and non-immune serum (N, dotted bar), immune sera or in immune sera containing the indicated complement inhibitors was determined as in Fig. 1. **B**. M3 streptococci were incubated in immune (I) and non-immune (N) sera. Covalently bound C3 and C4 fragments were then extracted from the bacteria and analyzed by SDS-PAGE (Stain) and immunoblot experiments with anti-C3 (Blot-C3) or anti-C4 (Blot-C4) Abs as probes. **C**. Multiplication of M3 bacteria in a mixture of neutrophils and non-immune serum (N, dotted bar), immune serum, or in C1qD-depleted immune serum reconstituted with buffer (depl),

C1q, factor D (FD), or both C1q and factor D was determined. **D**. Growth of M3 bacteria in a mixture of neutrophils and non-immune (N, dotted bar) or immune serum containing mAbs blocking the indicated complement components was determined as in Fig. 1. The data represent means and the range of 3–5 independent experiments. The statistical significance was assessed by the paired Student's *t*-test as indicated; * P < 0.05, ** P < 0.01, *** P < 0.001.

Figure 4. Induction of oxidative burst in neutrophils by *S. pyogenes* in immune serum is CD11b/CD18-dependent.

M3 streptococci were incubated for 30 min with human immune serum, neutrophils preloaded with the superoxide-sensitive dye DHR-123 and receptor-blocking mAbs (30 μ g/ml), or sCR1 (100 μ g/ml). Oxidative burst results in an increase in fluorescence as measured by flow cytometry. The mean fluorescence is indicated. Data representative of three separate experiments are shown.

Figure 5. Blocking of iC3b generation inhibits streptococcus-induced activation of neutrophil Cdc42 in immune human serum

Neutrophils were incubated with M3 bacteria in immune or non-immune serum, lysed at the indicated time points and GST-PAKcrib "pull-down" assays were performed. Proteins bound to GST-PAKcrib fusion proteins were analyzed by immunoblot experiments with anti-Rac or anti-Cdc42 Abs. In panels **A** and **B**, neutrophils were incubated with M3 bacteria in the presence of non-immune or immune serum (donor I). In panel **C**, neutrophils were incubated with M3 streptococci in the presence of serum from donor I with or without sCR1. The inset in panels A, B and, C are a representative blots. The values in each graph represent means \pm SEM of 5 - 8 independent experiments. The statistical significance of the differences was assessed by the paired Student's *t*-test; * P < 0.05.

Table 1. Critical role for CD11b/CD18 in killing of *S. pyogenes* by neutrophils in immune human serum.

				Multiplication factor ^[a]		P-value ^[e]	
Antigen	Antibody ^[b]	Serotype	Blocking ability ^[c]	Donor I	Donor II	Donor I	Donor II
Control	PBS			1.3 (0-9)	1.4 (0-8)		
CD11a	TS1/22[12]	IgG1	n.d. ^[d]	0.2 (0.1-0.4)	0.2 (0.1-0.5)	0.38	0.44
CD11b	44aacb[13]	IgG2a	42% +/- 5%	859 (675-1180)	388 (235-588)	< 0.01	< 0.01
CD11b	44aacb	$F(ab')_2$	n.d	498 (38-1329)	n.d ^[d]	< 0.01	-
HLA-ABC	W6/32	IgG2a	n.d.	0.7 (0-1)	n.d	0.63	-
CD11b	2LMP19c	IgG1	n.d	1039 (736-1470)	n.d	< 0.01	-
CD11c	3.9 [14]	IgG1	n.d	7 (4-9)	0.7 (0.3-1)	< 0.01	0.65
CD16	3G8 [15]	IgG1	69% +/- 10%	10 (4-20)	12 (130)	< 0.01	0.06
CD32	IV.3 [16]	IgG2b	50% +/- 10%	1 (0.1-3)	0.7 (0.5-0.8)	1.00	0.62
CD32	AT10 [17]	IgG1	41% +/- 10%	2 (0-4)	n.d.	0.79	-
CD35	3D9 [18]	IgG1	n.d	1 (0-3)	0.9 (0.1-1)	0.77	0.74
CD89	MIP7c [19]	IgG1	n.d	7 (0.2-10)	1 (0-2)	< 0.01	0.84

^[a] Mouse monoclonal antibodies (mAbs) blocking the indicated human neutrophil receptors were added at a final concentration of 30 μ g/ml, to a mixture of neutrophils, streptococci and immune serum. M3 bacteria were mixed with serum from donor I, and M1 bacteria with serum from donor II. The data represent means and the range of 3–5 independent experiments.

^[b] The mAbs MIP7c [19] (Absea Biotechnology), 3G8 [15] (Pharmingen), 2LMP19c and W6/32 (Dako) were purchased, while mAbs 3.9 [14] (Dr. N. Hogg), 3D9 [18] (Dr. E. J. Brown), and AT10 [17] (Dr. M. J. Glennie) were as kind gifts. MAbs TS1/22 [12] (Developmental Hybridoma Bank), 44aacb [13] and IV.3 [16] (American Type Culture Collection) were purified from cell cultures on protein A-Sepharose 4B.

^[c] The function-blocking ability of selected mAbs was determined in flow cytometry experiments by inhibiting the association between neutrophils and yeast particles opsonized with antigen-specific Abs (for the anti-FcR mAbs) or with serum. The percent of inhibition is given as compared to relevant isotype control mAbs and was calculated from three experiments.

^[d] Not determined.

^[e] The statistical significance of the differences in multiplication factor between the indicated Ab and the control was assessed by the paired Student's *t*-test.

Figure 1









Flourescence intensity

Figure 5

