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Effective Targeting of Pathogens to Neutrophils via Chimeric Surfactant Protein D/Anti-CD89 Protein

Paul J. Tacken,* Kevan L. Hartshorn,‡ Mitchell R. White,‡ Cees van Kooten,‡ Jan G. J. van de Winkel,§ Ken B. M. Reid,¶ and Joseph J. Batenburg‡*

Targeting of specific pathogens to FcRs on immune effector cells by using bispecific Abs was reported to result in effective killing of the pathogens, both in vitro and in vivo. Instead of targeting a specific pathogen to an FcR, we assessed whether a broad spectrum of pathogens can be targeted to an FcR using surfactant protein D (SP-D). SP-D is a collectin that binds a great variety of pathogens via its carbohydrate recognition domain. A recombinant trimeric fragment of SP-D (rfSP-D), consisting of the carbohydrate recognition domain and neck domain of human SP-D, was chemically cross-linked to the Fab’ of an Ab directed against the human FcoRI (CD89). In vitro, the chimeric rfSP-D/anti-CD89 protein enhanced uptake of Escherichia coli, Candida albicans, and influenza A virus by human neutrophils. Blocking of the interaction between rfSP-D/anti-CD89 and either the pathogen or CD89 abolished its stimulatory effect on pathogen uptake by neutrophils. In addition, rfSP-D/anti-CD89 stimulated killing of E. coli and C. albicans by neutrophils and enhanced neutrophil activation by influenza A virus. In conclusion, rfSP-D/anti-CD89 effectively targeted three structurally unrelated pathogens to neutrophils. (Col)lectin-based chimeric proteins may thus offer promise for therapy of infectious disease. *The Journal of Immunology, 2004, 172: 4934–4940.

The ability of bacteria, viruses, and fungi to become resistant to therapeutic drugs together with the continuous emergence of new pathogens call for new approaches to the treatment of infectious disease. Recently, the myeloid FcoRI (CD89) has been identified as an effective target molecule for bispecific Ab-mediated immunotherapy (1). CD89 is expressed exclusively in myeloid cells, including neutrophils, eosinophils, and monocytes/macrophages (2, 3). The expression of CD89 on neutrophils is induced by chemoattractants (4) and the inflammatory mediator TNF-α (5) and is increased on infiltrated lung neutrophils isolated from bronchoalveolar lavage fluid of cystic fibrosis patients with chronic lung infection (4). Upon stimulation, CD89 mediates both phagocytosis and induction of a respiratory burst (2, 3). Opsonization of pathogens with bispecific Abs consisting of an Ab against a specific pathogen coupled to an Ab against CD89 induces phagocytosis and killing of the pathogen by CD89-expressing neutrophils (6, 7). In addition, CD89 targeting results in enhanced clearance of Bordetella pertussis in the lungs of human CD89 transgenic mice (6). The anti-CD89 Ab used in the targeting studies, mouse mAb A77, binds CD89 at an epitope distinct from the ligand binding domain. Therefore, A77 binding to CD89 will not be hampered by high concentrations of Abs already present in serum/tissues. However, a major disadvantage of bispecific Abs is the fact that they only bind a narrow range of pathogens.

The present study evaluates the effectiveness of a surfactant protein D (SP-D)/anti-CD89 chimeric protein as an approach to targeting a broad spectrum of pathogens to CD89. SP-D is secreted into the airspaces of the lung by the respiratory epithelium and plays a role in pulmonary host defense. It is a member of the collectin family, which includes manninn-binding lectin, conglutinin, collectins 43 and 46, and SP-A. SP-D consists of an N-terminal domain, a collagenous domain, an alpha-helical coiled coil neck domain, and a C-terminal globular carbohydrate recognition domain (CRD), which binds carbohydrates in a calcium-dependent manner (for review, see Refs. 8–10). Via its CRD, SP-D binds to carbohydrate moieties on the surface of a great variety of pathogens. SP-D isolated from the lung consists of dodecamers or higher order oligomeric complexes, which are formed by association of trimers of the basic polypeptide chain at their N termini (8, 9). Binding of multimeric SP-D to microorganisms can stimulate or inhibit their uptake by phagocytes depending on the specific pathogen involved. Furthermore, binding often leads to aggregation of the microorganisms, which may help in their removal via mucociliary clearance (reviewed in Refs. 8, 9, and 11).

In theory, a chimeric protein consisting of a trimeric fragment of human SP-D and an mAb against CD89 should bind a broad range of pathogens and direct them to CD89 on phagocytic cells. In the present study we chemically cross-linked a recombinant trimeric fragment of human SP-D (rfSP-D) to the Fab’ of mAb A77. The resulting rfSP-D/anti-CD89 fusion protein markedly enhanced the uptake of influenza A virus (IAV), Candida albicans, and Escherichia coli by human neutrophils in vitro. Furthermore, rfSP-D/
anti-CD89 enhanced killing of *C. albicans* and *E. coli* by neutrophils in vitro killing assays and enhanced neutrophil activation by IAV.

**Materials and Methods**

**Materials**

The previously described hybridoma that produces the A77 mAb (mIgG1) directed against human FcεRI (CD64) (13) were provided by Medarex (Annandale, NJ). Production of rCD89 and Abs 2D11 and 7D7 (mouse IgG) peroxidase conjugate was purchased from Nordic (Tilburg, The Netherlands).

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**Recombinant SP-D production**

Production of rSP-D, consisting of eight Gly-X-Y repeats of the collagen non-collagenous Type Culture Collection, Manassas, VA) were prepared as described previously (17). In brief, rSP-D was expressed in *E. coli*, in which it was present in inclusion bodies, and was purified by a procedure involving denaturation and renaturation of inclusion body material, followed by gel and affinity chromatography. The purity of the rSP-D was checked by SDS-PAGE and subsequent Coomassie Blue staining and Western blotting.

**Production of rSP-D/anti-CD89 and rSP-D/anti-CD64**

The rSP-D was dialyzed against PBS buffer, or PBS, or in D-PBS containing various concentrations of rSP-D, (r)hSP-D, and/or rSP-D/anti-CD89. In experiments in which pathogens were incubated with both rSP-D/anti-CD89 and (r)hSP-D, both proteins were added to the pathogens simultaneously. Where indicated, 7D7, 2D11, anti-CD89 Fab', anti-CD89 Fab', and/or anti-CD64 Fab' were added to the pathogens as described previously (16).

**Isolation of human neutrophils**

Heparinized venous blood was drawn from healthy volunteers. Neutrophils were separated by Ficoll-Histoque (Sigma-Aldrich) to generate Fab', Fab' were added to rSP-D/sSMCC in a molar ratio of 1:2.1. The solution was incubated in the dark at room temperature for 3 h, followed by overnight incubation at 4°C. Subsequently, unbound sites were alkylated by adding iodoacetamide (Sigma-Aldrich) to a final concentration of 25 mM, followed by 30-min incubation at room temperature. The protein mixture was loaded onto a Superdex 200 column (50 ml bed volume; Pharmacia Biotech, Uppsala, Sweden), and fractions were collected and analyzed by SDS-PAGE and silver staining. Fractions containing unbound rSP-D or Fab' were discarded. Fractions containing coupled proteins were pooled and subjected to maltose-agarose affinity purification. The previously described hybridoma that produces the A77 mAb (mIgG1) directed against human FcεRI (CD64) (13) were provided by Medarex (Annandale, NJ). Production of rCD89 and Abs 2D11 and 7D7 (mouse IgG) peroxidase conjugate was purchased from Nordic (Tilburg, The Netherlands).

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**Production of rSP-D/anti-CD89 and rSP-D/anti-CD64**

The rSP-D was dialyzed against PBS buffer, or PBS, or in D-PBS containing various concentrations of rSP-D, (r)hSP-D, and/or rSP-D/anti-CD89. In experiments in which pathogens were incubated with both rSP-D/anti-CD89 and (r)hSP-D, both proteins were added to the pathogens simultaneously. Where indicated, 7D7, 2D11, anti-CD89 Fab', anti-CD89 Fab', and/or anti-CD64 Fab' were added to the pathogens as described previously (16).

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at 37°C at 400 rpm for 1 h (E. coli) or 2 h (C. albicans). Water was added to lyse neutrophils by hypotonic shock. This did not affect pathogen viability, as determined in control experiments with known numbers of pathogens. Serial dilutions of the samples were prepared in PBS. E. coli samples were plated on Luria-Bertoni agar (Sigma-Aldrich), whereas C. albicans samples were plated on Sabouraud dextrose agar plates (Sigma-Aldrich). CFUs were determined after incubation at 37°C for 24 h. Killing was expressed as: percent killing = 100 × [CFU from control wells (without neutrophils) − CFU from experimental wells]/[CFU from control wells (without neutrophils)].

Statistics

Unless indicated otherwise, statistical analysis of the data was conducted by ANOVA, whereupon, provided that the F-test indicated a significant difference (p < 0.05) among groups, comparisons with one particular condition were made with Dunnett’s test.

Results

Generation of rfSP-D/anti-CD89 and rfSP-D/anti-CD64

Chemical cross-linking of the rfSP-D to the Fab’ of A77 resulted in a mixture of proteins (Fig. 2A). Size separation by gel filtration, followed by affinity purification to isolate proteins containing a functional rfSP-D CRD domain, resulted in the isolation of cross-linked proteins with an apparent molecular mass of 100 kDa or more (Fig. 2, B–D). The cross-linked proteins stained positively for mouse IgG (Fig. 2C) and human SP-D (Fig. 2D). No bands were visible in control experiments in which sera from nonimmunized goat and rabbit were used or in experiments testing for cross-reactivity between the rabbit anti-hSP-D antiserum and anti-CD89 Fab’ and between the goat anti-(mouse IgG) peroxidase conjugate and rfSP-D (data not shown). Similar patterns were obtained upon generation of rfSP-D/anti-CD64 (data not shown).

Effect of rfSP-D/anti-CD89 on pathogen uptake by neutrophils

The uptake of three different pathogens at various concentrations of rfSP-D/anti-CD89 or rfSP-D is shown in Fig. 3. The rfSP-D/anti-CD89 significantly enhanced uptake of IAV by neutrophils (Fig. 3A), C. albicans (Fig. 3B), and E. coli K12 (Fig. 3C) compared with control samples, whereas no significant enhancement was seen with rfSP-D. Uptake of unopsonized C. albicans by neutrophils was relatively low compared with uptake of unopsonized IAV and E. coli K12; only 4% of neutrophils had ingested C. albicans at the end of the incubation (data not shown). For this reason, uptake of C. albicans by neutrophils was assessed by determining the number of fluorescent neutrophils, rather than mean neutrophil fluorescence. Stimulation of uptake of pathogens by neutrophils in the presence of rfSP-D/anti-CD89 was confirmed by fluorescence microscopy (data not shown).

Effects of anti-CD89 Fab’, anti-CD89 F(ab’)2, and rfSP-D/anti-CD64 on uptake of E. coli by neutrophils

To determine whether the rfSP-D/anti-CD89-mediated increase in pathogen uptake was not due solely to binding of its Fab’ to CD89, experiments were conducted to determine the effect of Fab’ or F(ab’)2 from mAb A77 (anti-CD89 Fab’ and anti-CD89 F(ab’)2, respectively) on uptake of E. coli K12 by neutrophils. In contrast to rfSP-D/anti-CD89, neither anti-CD89 Fab’ nor anti-CD89 F(ab’)2 stimulated uptake (Fig. 4).
To exclude the possibility that chimeric proteins of rSP-D and Fab’ would nonspecifically stimulate neutrophils, the effect of rSP-D/anti-CD64 on E. coli K12 uptake by neutrophils was studied. The rSP-D/anti-CD64 protein should not target pathogens to neutrophils, as described in Materials and Methods. As described in Materials and Methods. After incubation at 37°C, the mean neutrophil fluorescence was determined by FACS analysis, and the percentages of the control value were calculated. Data are the mean ± SEM of at least four experiments. Significant difference from control, as determined by ANOVA and Dunnett’s test: *p < 0.01.

Effect of blocking CD89 and CRD on rSP-D/anti-CD89-mediated pathogen uptake

SP-D binds carbohydrates on microorganisms via its CRD in a calcium-dependent manner (9). This binding can be abolished by chelation of calcium with EDTA or addition of sugars (24). To determine whether the rSP-D/anti-CD89-mediated increase in pathogen uptake by neutrophils required binding of the CRD of the rSP-D moiety to the pathogen, CRD-pathogen interaction was blocked by performing uptake experiments in the presence of either 100 mM maltose or 5 mM EDTA. The rSP-D/anti-CD89-mediated increase in pathogen uptake by neutrophils was significantly reduced by both maltose and EDTA (Table I). Therefore, blocking the interaction between rSP-D/anti-CD89 and CRD on the neutrophils blocked rSP-D/anti-CD89-mediated pathogen uptake.

Effect of rSP-D/anti-CD89 in combination with SP-D

In the presence of SP-D, uptake of IAV (20) and E. coli K12 (21) by human neutrophils has been reported to be increased, whereas uptake of C. albicans (25) by rat alveolar macrophages was reported to be decreased. Recombinant hSP-D optimally enhanced uptake of E. coli by human neutrophils at concentrations of 0.5–1 µg/ml (20), which is on the same order as SP-D levels found in human serum (26). We chose to determine the effect of rSP-D/anti-CD89 on E. coli and C. albicans uptake by neutrophils in the presence of SP-D at a concentration of 0.88 µg/ml. For experiments on IAV uptake by neutrophils, the effect of rSP-D/anti-CD89 was tested at different rhSP-D concentrations.

Uptake of IAV by neutrophils was increased to maximally 164 ± 20% by rhSP-D compared with unopsonized control samples. Simultaneous addition of 7.2 µg/ml rSP-D/anti-CD89 significantly increased IAV uptake by neutrophils at all rhSP-D concentrations tested (Fig. 5A), whereas no significant effect was seen with 7.2 µg rhSP-D. In the presence of 0.88 µg/ml hSP-D, the uptake of C. albicans was 252 ± 30% of that without opsonization. Opsonization with 0.88 µg/ml hSP-D in combination with 8 µg/ml rSP-D/anti-CD89 significantly increased uptake to 309 ± 26%, whereas opsonization with 8.88 µg/ml hSP-D stimulated uptake to a significantly lower extent than opsonization with 0.88 µg/ml hSP-D or with 0.88 µg/ml hSP-D plus 8 µg/ml rhSP-D/anti-CD89 (Fig. 5B). Microscopic analyses suggested that the decrease in uptake at higher hSP-D levels was due to the formation of large aggregates of C. albicans, which could not readily be taken up by the neutrophils (data not shown). Opsonization of E. coli with 0.88 µg/ml hSP-D had at best a minor effect on the uptake of the bacteria (Fig. 5C). The uptake with 0.88 µg/ml hSP-D was significantly increased by simultaneous addition of 2

<table>
<thead>
<tr>
<th>Pathogen Pathogen Uptake (% of unopsonized control)</th>
<th>IAV-Phil82</th>
<th>IAV-PR8</th>
<th>C. albicans</th>
<th>E. coli K12</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>196 ± 23</td>
<td>104 ± 2</td>
<td>309 ± 31</td>
<td>293 ± 28</td>
</tr>
<tr>
<td>Maltose</td>
<td>122 ± 4a</td>
<td>ND</td>
<td>129 ± 14c</td>
<td>143 ± 20f</td>
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<td>EDTA</td>
<td>102 ± 5c</td>
<td>ND</td>
<td>108 ± 5a</td>
<td>106 ± 2e</td>
</tr>
<tr>
<td>rCD89</td>
<td>ND</td>
<td>ND</td>
<td>156 ± 24f</td>
<td>180 ± 15</td>
</tr>
<tr>
<td>7D7</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>103 ± 6e</td>
</tr>
<tr>
<td>2D11</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>274 ± 56</td>
</tr>
</tbody>
</table>

*Freshly isolated human neutrophils were incubated with FITC-labeled IAV, C. albicans, and E. coli K12, as described in Materials and Methods. Experiments were performed in D-PBS (no addition) or in D-PBS containing 100 mM maltose, 5 mM EDTA, 4 µg/ml rCD89, 50 µg/ml 7D7, or 50 µg/ml 2D11. Pathogens were either unopsonized or opsonized with 7.2 µg/ml (in the case of IAV-Phil82 and IAV-PR8), 16 µg/ml (in the case of C. albicans), or 0.5 µg/ml (in the case of E. coli K12) rSP-D/anti-CD89. After incubation at 37°C, the mean neutrophil fluorescence or the number of fluorescent neutrophils was determined by FACS analysis. Data represent the mean neutrophil fluorescence (IAV-Phil82, IAV-PR8, and E. coli K12) or the number of fluorescent neutrophils (C. albicans) as a percentages of the unopsonized control value ± SEM of at least four experiments (except for IAV-PR8, which was tested in three experiments). The significant difference from D-PBS (no addition) was determined by ANOVA and Dunnett’s test. 

*p < 0.05.

†p < 0.01.
The effect of rSP-D/anti-CD89 on neutrophil activation by IAV was determined by assessing neutrophil H$_2$O$_2$ production. Although opsonization of IAV with rSP-D did not significantly affect neutrophil activation, opsonization with rSP-D/anti-CD89 resulted in a significant increase in H$_2$O$_2$ production compared with unopsonized IAV (Fig. 6A).

Classical CFU assays were performed with *C. albicans* and *E. coli* to assess whether rSP-D/anti-CD89 enhances killing of pathogens by neutrophils. The clinical isolate *E. coli* ATCC 25922 was used for killing experiments, as the *E. coli* K12 strain used in the uptake experiments did not survive the hypotonic shock required to lyse neutrophils (data not shown). After 2 h of incubation, 21 ± 4% of the unopsonized *C. albicans* were killed. Opsonization with rSP-D did not significantly enhance killing, whereas killing was significantly increased by opsonization with rSP-D/anti-CD89 (Fig. 6B). One-hour incubation of *E. coli* with neutrophils resulted in 15 ± 2% killing. Similar to the results obtained for *C. albicans*, opsonization of *E. coli* with rSP-D did not significantly affect killing, whereas killing was significantly increased by opsonization with rSP-D/anti-CD89 (Fig. 6B).

**Discussion**

The implementation of Abs in clinical therapy has grown rapidly over the last 2 decades. Abs now represent >30% of biopharmaceuticals in clinical trials and are evaluated for treatment of cancer, autoimmune diseases, transplant rejection, and infectious diseases (27). Bispecific Abs are powerful tools for redirection of immune
effector cells to specific targets. FcRs on immune effector cells have been recognized as efficient trigger molecules for bispecific Ab-mediated killing of both tumor cells and pathogens (1, 28–30).

As a novel approach for treatment of infectious disease, we use the ability of SP-D to recognize a wide variety of microorganisms as a means to target pathogens to CD89. The rSP-D/anti-CD89 chimeric protein effectively targets IAV, *E. coli*, and *C. albicans* to neutrophils, leading to pathogen internalization. In contrast to rSP-D/anti-CD89, a molecule lacking the rSP-D moiety (anti-CD89 Fab’), a molecule containing a different Fab’ (rSP-D/anti-CD64), and a molecule lacking the Fab’ (rSP-D) do not stimulate uptake of *E. coli* by neutrophils. In addition, pathogen targeting to neutrophils is blocked when EDTA or maltose hampers the CRD-pathogen interaction, or when rSP-D/anti-CD89 binding to the neutrophil CD89 is reduced by rCD89 or CD89-blocking Ab. Moreover, rSP-D/anti-CD89 does not stimulate uptake of the IAV PR8 strain, which has been described not to bind SP-D. These findings indicate that rSP-D/anti-CD89 functions as it was designed to do, namely, stimulating pathogen uptake by binding to the pathogen via its rSP-D CRD domain and by binding CD89 via its Fab’ domain.

With regard to the blocking experiments with EDTA, it is relevant to note that in some of our experiments the level of uptake in EDTA control samples differed from that in samples without EDTA. Depending on the pathogen, EDTA increased, decreased, or had no effect on uptake levels. However, uptake was never completely blocked (data not shown). Apparently, uptake of pathogens by neutrophils can largely continue in the presence of EDTA. This resembles the observations by Della Bianca et al. (31), who observed that phagocytosis of *Sacharomyces cerevisiae* by neutrophils was not, or was only partly, inhibited by calcium depletion depending on the receptors involved. Signal transduction upon triggering of CD89 on neutrophils was found to depend on release of calcium from an intracellular store that is not affected by extracellular addition of a calcium chelator (32). Therefore, the inhibition by EDTA of the stimulatory effect of rSP-D/anti-CD89 on pathogen uptake by neutrophils (Table I) is probably not due to a blockage of CD89-dependent uptake per se, but, rather, to blockage of binding of the rSP-D moiety to the pathogens.

SP-D by itself has been reported to stimulate neutrophil uptake of IAV (20) and *E. coli* (21). However, our data indicate that at SP-D concentrations found in human serum (26), pathogen uptake by neutrophils by neutrophils is more effectively stimulated by addition of rSP-D/anti-CD89 than by SP-D. The trimeric rSP-D protein did not significantly increase pathogen uptake by neutrophils at any of the concentrations used in our studies.

In addition to increasing uptake, rISP-D/anti-CD89 enhances killing of both *E. coli* and *C. albicans* by human neutrophils in vitro. With regard to IAV, targeting to neutrophils is likely to incapacitate the virus, as IAV does not replicate in neutrophils (33), whereas rISP-D/anti-CD89 augments the activation of neutrophils by IAV (Fig. 6A), thus increasing their capacity to neutralize the virus.

SP-D binds to an overlapping, but distinct, range of pathogens compared with other collectins and lectins. Together, the (col)lectins cover a broad spectrum of viral, fungal, and bacterial pathogens and could be useful tools in immunotherapy. As SP-D is highly expressed in the lung, FcR targeting with SP-D-based chimeric proteins might prove useful in the treatment or prevention of pulmonary infections. In addition, the chimeric proteins could be applied systemically. A similar approach, using i.v. administration of bispecific Abs directed against *C. albicans* and human FcγRI (CD64), has been shown to effectively protect CD64 transgenic mice from lethal invasive candidiasis (29).

In its present form, rISP-D/anti-CD89 might trigger an immunogenic reaction in humans, as it contains a murine Fab’. This should be overcome by cross-linking rISP-D, which has the human amino acid sequence, to a fully human anti-CD89 Ab. The rSP-D part can be expected to be nonimmunogenic, as SP-D is present in blood (26).

CD89 is expressed on neutrophils and various types of macrophages (34). This expression pattern makes it a suitable trigger molecule for cell-mediated killing (1). Most likely, i.v. application of rISP-D/anti-CD89 would lead to targeting of pathogens to neutrophils and monocytes in blood, whereas intranasal application would lead to targeting alveolar macrophages and infiltrated neutrophils. As an alternative for CD89, targeting to CD64 might be preferable when the objective is to induce antigenic memory. CD64 is not constitutively expressed on neutrophils, but is expressed on APCs such as monocytes/macrophages and dendritic cells (23, 35). Furthermore, targeting of weak Ags to CD64 elicits potent humoral responses and has been reported to induce immunological memory in human CD64 transgenic mice (29, 36). Although CD89 is expressed on monocytes/macrophages, reports on whether CD89 is expressed on dendritic cells contradict each other (37, 38). Thus, it remains to be determined whether targeting CD89 induces humoral responses as potently as targeting CD64.

There are many cell surface molecules that could be used as a target for immunotherapy. Future studies will have to reveal which target molecule gives the best results for each individual targeting strategy.

In conclusion, the present paper describes highly effective targeting of three structurally unrelated pathogens to immune effector cells using a (col)lectin/anti-FcR chimeric protein. The strategy of combining the broad pathogen recognition ability of (col)lectins with the key role that FcRs play in host defense opens up new ways for treatment and prevention of infectious disease.

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**References**


