Soluble M1 protein of *Streptococcus pyogenes* triggers potent T cell activation

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Summary

*Streptococcus pyogenes* of the M1 serotype is commonly associated with large outbreaks of invasive streptococcal infections and development of streptococcal toxic shock syndrome (STSS). The pathogenesis behind these infections is believed to involve bacterial superantigens that induce potent inflammatory responses, but the reason why strains of the M1 serotype are over-represented in STSS is still not understood. In the present investigation, we show that a highly purified soluble form of the M1 protein from *S. pyogenes*, which lacks the membrane-spanning region, is a potent inducer of T cell proliferation and release of Th1 type cytokines. M1 protein-evoked T cell proliferation was HLA class II-dependent but not MHC-restricted, did not require intracellular processing and was Vβ-restricted. Extensive mass spectrometry studies indicated that there were no other detectable proteins in the preparation. Taken together, our data demonstrate that soluble M1 protein is a novel streptococcal superantigen, which likely contributes to the excessive T cell activation and hyperinflammatory response seen in severe invasive streptococcal infections.

Introduction

Streptococcal toxic shock syndrome (STSS), caused by the human pathogen *Streptococcus pyogenes*, is a severe and life-threatening condition characterized by hypotension, tissue destruction and multiple organ failure (McCormick et al., 2001). Although the pathogenesis behind STSS is not fully understood, bacterial superantigens are believed to play an important role. Superantigens bind, without prior cellular processing, to MHC class II on antigen-presenting cells and to the Vβ chain of the T cell receptor (TCR), thereby activating a large pool of T lymphocytes independently of normal antigen processing and presentation (Marrack and Kappler, 1990). While a regular antigen activates approximately 0.01% of the T cell population, superantigens may activate up to 20% of the resting T cell population (McCormick et al., 2001). The massive immune activation leads to an excessive production of pro-inflammatory cytokines, which is believed to be responsible for many of the severe systemic effects seen in STSS patients, such as vasodilation and multiple organ failure. Although great advances have been made in intensive care medicine over the last years, STSS is still a feared condition due to its extremely rapid progression and high mortality rates (Stevens et al., 1989; Darenberg et al., 2007). However, administration of human polyspecific immunoglobulin (IVIG) has lately been reported to have beneficial effects in the treatment of severe infections caused by *S. pyogenes* (Kaul et al., 1999; Darenberg et al., 2003; Norrby-Teglund et al., 2005), and important mechanisms of action are believed to be the neutralization of superantigens and dampening of the hyperinflammatory response (Norrby-Teglund et al., 1996a; Norrby-Teglund et al., 2003).

So far, 12 superantigens have been characterized in *S. pyogenes*, including the streptococcal pyrogenic exotoxins (Spe) A–M, the streptococcal superantigen (SSA) and the streptococcal mitogenic exotoxin (SmeZ) 1 and 2 (Proft et al., 1999; Proft and Fraser, 2003). In addition, a pepsin-cleaved fragment of M proteins from streptococcal strains associated with acute rheumatic fever, such as the M5 serotype, has been demonstrated to trigger T cell proliferation (Tomai et al., 1992; Watanabe-Ohnishi et al., 1994; Pérez-Lorenzo et al., 2006). M proteins, which are surface proteins of *S. pyogenes*, have an α-helical coiled coil structure with a conserved C-terminal part and a
highly variable N-terminal end that defines the M serotype (for review, see Fischetti, 1989). More than 150 different M proteins have been characterized to date, and certain types are more commonly seen in association with specific disease manifestation. Epidemiological studies have revealed that the M1 serotype is commonly associated with large outbreaks of severe invasive streptococcal infections, such as STSS (Davies et al., 1996; Cunningham, 2000), but the underlying rationale is, as of yet, not fully elucidated. M proteins are normally anchored to the bacterial cell wall via an LPXTG motif, but they can also be released from the surface via proteases derived from the host or from the bacterium itself (Åkesson et al., 1994; Berge and Björck, 1995; Herwald et al., 2004). The M protein has long been regarded as an important virulence factor of *S. pyogenes* due to its antiphagocytic properties (Lancefield, 1962). Recently, however, it was shown that soluble M protein of the M1 serotype also induces innate immunity by activating neutrophils (Herwald et al., 2004) and monocytes (Påhlman et al., 2006). In the present study, we investigated the effect of soluble M1 protein on T cell activation and cytokine production, which are pronounced features of STSS.

### Results

M1 protein is a potent inducer of proliferation in peripheral blood mononuclear cells

In order to study the effect of M1 protein on T cell proliferation, peripheral blood mononuclear cells (PBMC) were stimulated with various concentrations of M1 protein for 72 h. As seen in Fig. 1A, M1 protein evoked a powerful and dose-dependent proliferative response that was highly reproducible within the same donor (Fig. 1A). The level of response to M1 protein varied when cells from different individuals were used as responders (Fig. 1B). However, M1 protein-evoked proliferation was in all cases similar to that evoked by a superantigen-containing supernatant prepared from overnight cultures of the M1 strain AP1, in the following referred to as AP1 Sup (Fig. 1B). In contrast, no proliferation was induced by M49 protein (Fig. 1B).

The M1 protein used in this study was purified from an isogenic AP1 mutant strain that expresses a truncated M1 protein lacking the membrane-spanning region. Consequently, the streptococcal protein accumulates in the growth medium, from which it is purified based on its ability to bind human fibrinogen with high affinity (Kantor, 1965; Åkesson et al., 1994). M1 protein is the only fibrinogen-binding protein of the AP1 strain (Berge and Björck, 1995; Collin and Olsén, 2000), and although the purified protein was pure as judged by Coomassie and silver staining, the concern of whether the T-cell mitogenic effects of M1 protein could be due to trace amounts of other contaminating superantigens was addressed. Most of the streptococcal superantigens are encoded by phage-mediated genes, and each bacterial strain therefore expresses its own repertoire of superantigens (Llewelyn and Cohen, 2002). Genotyping of the AP1 strain showed that it carried the genes coding for SpeA, B, F, G, J, and SmeZ, but not for SpeC, H, or SSA. As the mutant soluble M1 protein has a molecular weight (MW) of 40 kDa, whereas all known streptococcal superantigens have a size of 24–29 kDa, the M1 protein preparation was further separated based on size by fast protein liquid chromatography (FPLC). FPLC fractions containing the soluble M1 protein retained mitogenic activity on T lymphocytes (42.823 ± 1.585 cpm).

The M1 protein preparation was subjected to two different matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry analyses, including the seed-layer sample technique for visualization of full-length M1 protein as well as the ‘salami-method’ using
SDS-PAGE separated M1 protein. The full-length M1 protein demonstrated two distinct peaks representing the M1 protein at its different charges (Fig. 2A). Importantly, no other signals were demonstrated including the area spanning the 20–30 kDa range, i.e. the area of potentially contaminating streptococcal superantigens. To ensure that a superior signal of the M1 protein did not shield any contaminating proteins, the M1 protein was separated on SDS-PAGE and sequential gel pieces starting from the M1 protein and downwards in falling molecular weight were excised and analysed in parallel. M1 protein was identified with a highly significant score ($P < 0.05$) in the two top excised gel bands (data not shown). No other proteins could be identified here or in any of the subsequent gel pieces using the Mascot search engine and the probability-based Mowse score. Moreover, no specific peptides deriving from the SmeZ protein were found with the Expasy FindPept tool, using strict mass-error and cleavage accuracy criteria.

To further test the purity of the M1 protein, human anti-M1 protein antibodies were isolated from an IVIG preparation shown to contain high titres of antibodies against M proteins (Basma et al., 1998). IVIG was adsorbed to M1 protein-coupled Sepharose. To ensure purity, we used recombinant M1 protein generated in Escherichia coli to obtain the anti-M1 antibodies and avoid any contamination with anti-superantigen antibodies. Also, to avoid contamination by non-specific binding of IgG, the sepharose was washed with a sodium citrate buffer of pH 5.5 prior to elution of anti-M1 protein antibodies. As shown in Fig. 2B, the unfractionated IVIG preparation neutralized the mitogenic activity of both M1 protein and the

![Fig. 2. Specificity of M1 protein-induced responses. To test the purity of the M1 protein preparation full-length unseparated M1 protein was analysed by mass spectrometry. A. MALDI-TOF mass spectrometry spectrum of the full-length M1 protein. The designated peaks represent the M1 protein at its different charges. The calibrated MW is 38 155 kDa. Note the absence of signals in the 20–30 kDa range. B. PBMC were stimulated with M1 protein (1 μg ml$^{-1}$, white bars) or AP1 Sup (1:200 dilution, black bars) in the absence or presence of anti-M1 protein antibodies (0.1 mg ml$^{-1}$) purified from polyspecific immunoglobulin (IVIG), or IVIG (2.5 mg ml$^{-1}$). Proliferation was detected by $^3$H-thymidine uptake after 72 h of stimulation. The figure shows the inhibitory effect of anti-M1 protein and IVIG on proliferation induced by M1 protein or AP1 Sup. Data represent the mean and SD of four independent experiments. The Mann–Whitney U-test was used to evaluate statistical differences in antibody-mediated inhibition of M1 protein as compared with the AP1 Sup ($P = 0.0159$). C. M1 protein prepared from either streptococcal MC25 supernatant (a) or in E. coli (b) was analysed by negative staining and electron microscopy. Arrows indicate the elongated shape of streptococcal M1 protein and arrowheads the globular shape of E. coli-expressed recombinant M1 protein. Size bar = 100 nm. D. PBMC incubated in the presence (triangles) or absence (squares) of the recombinant M1 protein fragment A–S (1 μg ml$^{-1}$) prior to stimulation with M1 protein (100 ng ml$^{-1}$) or with AP1 supernatant (1:100 dilution). Proliferation was assessed as described above, and the figure shows matched results of individual experiments. The horizontal lines denote median values. © 2007 The Authors Journal compilation © 2007 Blackwell Publishing Ltd, Cellular Microbiology, 10, 404–414]
AP1 Sup. In contrast, the pure anti-M1 protein antibodies significantly inhibited the mitogenic activity of M1 protein, but had no effect on the AP1 Sup \( (P = 0.0159) \) (Fig. 2B).

The recombinant M1 protein was also tested for mitogenic activity, but did not induce any proliferation (data not shown). It cannot be excluded that the lack of activity of the recombinant M1 protein is attributed to differences in post-translational modifications in \textit{E. coli} compared with \textit{Streptococcus}. To further assess this, we analysed the recombinant and native M1 protein with electron microscopy, which revealed drastic differences in structure between the two preparations (Fig. 2C). The streptococcal M1 protein had the correct size of approximately 50 nm and demonstrated the expected elongated form consistent with the M proteins being \( \alpha \)-helical coiled-coil dimers. The native M49 protein demonstrated a highly similar structure as the M1 protein (data not shown). In contrast, the recombinant M1 protein had a globular structure, and consequently, it seems reasonable to assume that the lack of mitogenic activity by the recombinant M protein is due to this structural difference.

To further test the specificity of the M1 protein-induced responses, we prepared a recombinant fragment covering the N-terminal part of the M1 protein and used this in blocking experiments. Pre-incubation of cells with the fragment caused a marked inhibition of the M1 protein induced proliferation, whereas the AP1 supernatant remained unaffected (Fig. 2D). Taken together, these results show that the proliferative responses are attributed to the M1 protein and not due to any contamination with other superantigens.

\textbf{M1 protein induces T lymphocyte proliferation in a HLA class II-dependent manner}

The potent effect of M1 protein on T cell proliferation prompted us to investigate whether the streptococcal protein itself could act as a superantigen. To determine the influence of HLA class II on T cell activation in response to M1 protein, we used mitomycin C-treated HLA class II-negative BLS cells or BLS cells transfected with various HLA class II alleles as antigen-presenting cells, and co-cultured those with purified T cells in the absence or presence of M1 protein. The M1 protein-induced T cell responses were significantly higher in cultures with HLA class II-transfected BLS cells than with untransfected \( (P = 0.03; \) Fig. 3), demonstrating that HLA class II is required to obtain efficient T cell activation by M1 protein. As a control, cells were stimulated with the superantigen-containing AP1 Sup, which demonstrated a similar HLA class II dependency as previously reported for streptococcal superantigens (Norrby-Teglund \textit{et al.}, 2002). In contrast, all BLS cell lines, untransfected or transfected, promoted equally high PHA responses (data not shown). Taken together, these experiments demonstrate that M1 protein-induced T cell proliferation does not require cellular processing of the protein, and that it is HLA class II-dependent but not MHC-restricted.

\textbf{V\delta profile of M1 protein}

The major hallmark of a superantigen response is the preferential activation of T cells bearing specific subsets of V\delta regions. Superantigens normally have affinity for a defined set of V\delta chains, which gives each superantigen a unique V\delta profile. In order to analyse whether the T cell proliferation induced by M1 protein was V\delta-restricted, the V\delta specificity of T cells stimulated with M1 protein was determined by flow cytometric analyses. Comparison between frequencies of T cells with specific V\delta elements in cultures stimulated with M1 protein and PHA, revealed a consistent pattern of preferential expansion of V\delta2- and V\delta4-expressing T cells in response to M1 protein (Fig. 4). Although expansion of V\delta8 and V\delta22 could be observed in some individuals, it was not a consistent finding and did not reach statistical significance. Taken together, the data demonstrate that T cell activation by M1 protein is TCR V\delta-restricted, which is characteristic for superantigen activity.

\textbf{M1 protein induces a Th1 type of cytokine response}

As superantigens are known to predominantly induce Th1 type of cytokines (Norrby-Teglund \textit{et al.}, 1994a; 1997), we
next analysed cytokine responses triggered by M1 protein. To that end, PBMC were treated with M1 protein, AP1 Sup, or medium alone for 72 h, and TNFβ- and IFNγ-producing cells were identified by intracellular immunohistochemical staining. Virtually no positive cells were detected in unstimulated cells, whereas a mean of 1.9% of the cells expressed TNFβ and 4.2% expressed IFNγ in response to M1 protein (Fig. 5A). Similar to the proliferation experiment, a large degree of inter-individual variation was noted when PBMC from different individuals were used. Similar frequencies were detected in cells stimulated with the superantigen-containing AP1 Sup (Fig. 5A). However, when cells were incubated with M1 protein in the presence of IVIG, the secretion of TNFβ and IFNγ was almost completely abolished (Fig. 5B and C).

The marked Th1 response and the large number of cytokine-producing cells in response to M1 protein provide further evidence for a superantigen effect of M1 protein.

Discussion

The M protein has recently received a lot of attention due to its immunostimulatory effects, particularly in its soluble form. Soluble M1 protein was found to be a powerful inducer of neutrophils (Herwald et al., 2004) as well as of monocytes (Påhlman et al., 2006). In the present study, we demonstrate yet another property of soluble M protein from the M1 serotype as a potent T cell activator, inducing pronounced T cell proliferation and production of Th1 type of cytokines. Several publications have demonstrated that the severe invasive streptococcal infections are characterized by excessive pro-inflammatory responses, including the classical sepsis cytokines IL1, IL6, IL8 and TNFα, but also T cell-derived cytokines predominantly of Th1 type, i.e. TNFβ and IFNγ (Norrby-Teglund et al., 2000a; 2001). Superantigens have been implicated as central mediators of these pathologic cytokine responses and consequently the systemic effects seen in severe invasive

Fig. 4. M1 protein induces expansion of Vβ-specific T cells. PBMC were stimulated with M1 protein or PHA for 72 h, after which cells were stained with fluorescently labelled antibodies against the T cell marker CD3 and a panel of 24 different human Vβ subclasses, followed by flow cytometric analysis. T cells were identified by gating on the lymphocyte population based on FSC/SSC characteristics and CD3 expression. In this T cell population, the number of M1 protein-treated cells positive for each Vβ family was counted and compared with the number of PHA-treated cells of the same Vβ class. Wilcoxon signed rank test was used for paired analyses of M1-stimulated cells and PHA-stimulated cells (*P = 0.03).

Fig. 5. M1 protein induces a Th1 type of cytokine response. PBMC were stimulated with various stimuli for 72 h, and stained for intracellular cytokines as described in Experimental procedures.

A. The amount of cells positive for TNFβ (squares) or IFNγ (triangles) in response to M1 protein (1 µg ml⁻¹) or AP1 Sup (1:50 dilution). Horizontal lines represent the mean of six separate experiments. No or only very low frequencies of cytokine producing cells (TNFβ: 0.03 ± 0.08%; IFNγ: 0.1 ± 0.1% positive cells) could be detected in unstimulated cells.

B and C. Cells expressing TNFβ (B) or IFNγ (C) after stimulation with M1 protein (1 µg ml⁻¹) in the absence or presence of IVIG (2.5 mg ml⁻¹). Statistically significant differences between paired samples were evaluated by use of the Wilcoxon signed rank test (*P = 0.03).
S. pyogenes infections (Miethke et al., 1992; Bonventre et al., 1993; Watanabe-Ohnishi et al., 1995; Stevens et al., 1996; Norby-Teglund et al., 2001). However, it has not been possible to link a particular superantigen with development of STSS. Our finding that soluble M1 protein is equally potent as other streptococcal superantigens in the induction of T cell activation and cytokine production, together with our previous observation that shed M1 protein is present in tissue biopsies from patients infected with M1T1 S. pyogenes (Herald et al., 2004), strongly implies that M1 protein contributes to the excessive cytokine response seen in these infections.

The potency of M1-induced T cell activation warranted further studies in order to elucidate whether the protein acted as a superantigen. To this end, we analysed the requirement for MHC class II molecules as well as the major superantigen definition criteria of preferential expansion of T cells bearing certain Vβ elements. The studies revealed that M1 protein-induced activation displayed all the characteristics of a superantigen, namely powerful T cell activation without cellular processing of the protein, HLA class II dependency, no MHC restriction, T cell activation in a Vβ-specific manner, and the mounting of a Th1 response in a large number of T lymphocytes. The experiments revealed a substantial inter-individual variation in M1 protein-induced responses. This has previously been reported for superantigen-induced responses and the variation seems to result from variations in the individuals TCR Vβ repertoire and HLA class II molecules (Norby-Teglund et al., 1994a; 2002; Kotb et al., 2002).

As S. pyogenes produces a vast number of superantigens, a major concern in studies such as this one is that the noted response could be due to trace amounts of contaminating superantigens. Each superantigen activates T cells in a Vβ-specific manner, and the Vβ profile of the superantigen may therefore be used as a fingerprint of that protein. The M1 protein was found to induce preferential expansion of T cells expressing Vβ2 and Vβ4, which is a unique profile previously not reported for any other streptococcal superantigen. Although both Vβ2 and 4 are part of the Vβ specificity of several superantigens, including SmeZ, SpeJ and SpeC, the complete Vβ profile of respective superantigen is each one unique as they include additional Vβ elements. Interestingly, Vβ4 seems to be shared among pepsin-extracted (pep) M proteins of rheumatogenic serotypes, which have been reported to act as superantigens (Tomai et al., 1990; 1991; Watanabe-Ohnishi et al., 1994). The superantigenicity of the M protein has been an area of debate for many years and it has been argued that the rheumatogenic pepsin-digested (pep) M proteins have been contaminated with other superantigens (Degnan et al., 1997). Evidence that the superantigenicity was indeed mediated by pepM5 was provided by the finding that M5-specific peptides preferentially blocked the expansion of pepM5-specific Vβ elements (Wang et al., 1993). In the current study, a number of experiments have been conducted to ensure that the effects are M1 protein-specific, including extensive mass spectrometry that failed to detect any known superantigens. Most compelling, however, was the fact that the anti-M1 protein antibodies significantly neutralized the M1 protein-induced T cell activation, whereas no effect was seen on the superantigen-containing bacterial supernatant. Similarly, a recombinant N-terminal M1 fragment could potently inhibit the M1 protein-induced proliferation, whereas the superantigen-containing supernatant remained unaffected. Hence, our data show that soluble M1 protein is a novel superantigen of S. pyogenes.

We have focused on the M protein from the serotype M1T1, in an attempt to advance our understanding of the significant association between infection with this serotype and the development of STSS, as well as increased fatality rates (Strömberg et al., 1991; Davies et al., 1996; Zurawski et al., 1998). Our finding that soluble M1 protein is a potent T cell activator is likely to contribute to the excessive cytokine responses seen in STSS cases; however, whether this is a unique property of the M1 protein remains to be shown. In this study, we have made some initial observations with soluble M49 protein, which failed to induce significant proliferative responses. The M49 serotype has been associated with post-streptococcal glomerulonephritis and has not been linked to severe invasive streptococcal infections. However, further studies are required in order to define the link between the immunostimulatory activity of M proteins activity and association to severe systemic infections.

The recent finding that M1 protein triggers innate immunity through activation of monocytes via Toll-like receptor (TLR) 2 (Pålman et al., 2006) may also contribute to the T cell activation seen here. It is known that activated T cells upregulate TLR2 on their surfaces, where it functions as a costimulatory receptor that enhances cytokine production in response to activation by an anti-CD3 antibody (Komai-Koma et al., 2004). Moreover, the TLR4 agonist LPS has been reported to enhance superantigen toxicity by 50 000 times in a rabbit model of toxic shock syndrome (Schleifer, 1982), and it is therefore tempting to speculate that the TLR2 agonistic properties of the M1 protein may also contribute to its potent effect on T cells. In a clinical setting also other TLR agonists, such as lipoteichoic acid and peptidoglycan, are likely to contribute to the overall inflammatory response.

Aside from bacterial strain characteristics, variations in the host response to superantigens seem to influence the outcome of a streptococcal infection. Kotb et al. (2002) have shown that certain HLA haplotypes are associated with an increased risk of severe streptococcal disease, and several studies have demonstrated that lack of
protective antibodies against superantigens and against the M protein is a risk factor for the development of STSS (Norrby-Teglund et al., 1994b; Basma et al., 1999). The latter findings may explain the beneficial effects of IVIG in the treatment of STSS, as IVIG contains antibodies against a wide array of streptococcal proteins, including superantigens and M proteins (Norrby-Teglund et al., 1996b; 2000b; Basma et al., 1998). Although M proteins render the streptococcus resistant to phagocytosis in the non-immune host, the bacterium is efficiently cleared in the presence of type-specific antibodies against M protein (Lancefield, 1962), and anti-M protein antibodies in IVIG preparations have been demonstrated to facilitate clearance of bacteria via opsonization (Basma et al., 1998).

The results in the present study suggest yet another role for antibodies against M protein in the treatment of invasive streptococcal disease, namely to neutralize the T cell mitogenic effect of soluble M protein. Taken together, the present investigation demonstrates a novel mechanism by which M1 protein contributes to the virulence of *S. pyogenes*. It further emphasizes the role of M1 protein in the pathogenesis of streptococcal infections and may help explain why streptococci of the M1 serotype are over-represented in STSS.

**Experimental procedures**

**Purification of the M1 protein**

M1 protein was purified by adsorption to fibrinogen-coupled sepharose as previously described from the growth medium of MC25, an isogenic mutant strain derived from the AP1 *S. pyogenes* strain 40/58 (from the World Health Organization Collaborating Centre for references and Research on Streptococci, Institute of Hygiene and Epidemiology, Prague, Czech Republic) (Collin and Olsén, 2000). The MC25 mutant expresses an M1 protein that lacks the membrane-spanning region, resulting in a soluble protein that is secreted into the growth medium. The purity of the M1 protein preparation was confirmed by SDS-PAGE analysis followed by Coomassie or silver staining, as well as by mass spectrometry as described below. The M1 protein was also exposed to FPLC purification based on size. Two hundred microlitres of the M1 protein purification was loaded on a Superose 12 column (Pharmacia, Uppsala, Sweden) and washed through with PBS. Collected fractions were analysed on SDS-PAGE, and M1 protein-containing fractions were pooled. Recombinant M1 protein and the N-terminal part of M1 protein, fragment A–S, were produced as described elsewhere (Åkesson et al., 1994). M49 protein was obtained from *S. pyogenes* bacteria of an M49 strain. Surface proteins were solubilized by CNBr cleavage, and M49 protein was purified on fibrinogen-coupled sepharose as above.

**Electron microscopy**

M1 protein purified from supernatant from the streptococcal strain MC25, the streptococcal M49 protein and the recombinant M1 protein expressed in *E. coli* were analysed by negative staining and electron microscopy as described previously (Engel and Furthmayr, 1987). Aliquots (5 µl) were adsorbed onto carbon-coated grids for 1 min, washed with two drops of water and stained on two drops of 0.75% uranyl formate. The grids were rendered hydrophilic by glow discharge at low pressure in air.

Specimens were observed in a Jeol JEM 1230 electron microscope operated at 60 kV accelerating voltage. Images were recorded with a Gatan Multiscan 791 CCD camera.

**Determination of M1 protein purity by MALDI-TOF mass spectrometry**

The M1 protein preparation was subjected to two different analyses. For visualization of full-length M1 protein and an indication of purity with regard to the 20–30 kDa MW ranges, the seed-layer sample preparation technique was used.

Briefly, matrix [20 mg ml\(^{-1}\) sinapinic acid in 50% acetonitrile (ACN) and 0.1% triflouric acid (TFA)] was applied to a stainless steel sample target plate and air-dried. M1 was diluted first in 0.1% TFA to 10 pmol µl\(^{-1}\) and then with matrix 1:1. After application to the sample target and drying, the obtained spot was washed three times with cold 0.1% TFA.

The target plate was loaded into a Bruker Reflex™ III MALDI-TOF mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany). Polarity of the instrument was set for positive ions and a calibrated spectrum was obtained with a method optimized for the MW range under investigation.

To obtain a separation of the M1 sample, SDS-PAGE and Coomassie staining was performed. Afterwards, six gel pieces were cut out in sequence starting at a distinctly stained M1 protein band and downwards in falling MW order (the salami method). Note that no additional bands were visible in the lane subject to investigation, but gel pieces processed anyhow. Samples were washed four times with 40% (v/v) ACN. They were evaporated to dryness and reduction of proteins was carried out in 10 mM dithiothreitol at 56°C for 30 min. Superfluous solution was aspirated and proteins were alkylated with 30 mM iodoacetamide. The gel pieces were washed as above and again evaporated to dryness. Potential proteins were then degraded into characteristic fragments with trypsin (Porcine, sequencing grade, Promega). Briefly, gel pieces were re-hydrated in 25 µg ml\(^{-1}\) enzyme solution (in 25 mM NH\(_4\)HCO\(_3\)) and incubated for 16 h at 37°C.

After extraction of gel pieces with 2% TFA, peptide-containing solutions were purified and concentrated using C18 ZipTip™ (Millipore) according to the manufacturer’s instructions. Samples were applied to a MALDI AnchorsChip™ target plate pre-spotted with 2,5-dihydroxybenzoic acid (DHB) matrix, which was then loaded into the MALDI-TOF mass spectrometer. Polarity of the instrument was set for positive ions with delayed extraction and the detector for reflector mode. The acceleration voltage was 25 kV and 75–100 shots per sample were summed in each spectrum for improved signal-to-noise ratio. Spectra were internally calibrated using trypsin autolysis fragments. These were then analysed for protein identifications (peptide mass fingerprinting) from primary sequence databases using the Mascot search engine (http://www.matrixscience.com). Additionally, the Expasy server FindPept tool (http://www.expasy.ch) was used to search for experimentally obtained peptide masses matching with the trypsin cleavage pattern of the potential sample contami-
Preparation of superantigen-containing bacterial culture supernatant

AP1 bacteria were grown in Todd Hewitt broth (BD, Franklin Lakes, NJ) in 5% CO₂ at 37°C overnight. Bacteria were then pelleted by centrifugation, and 5 ml of the culture supernatant was mixed with 20 ml of ice-cold EtOH. Proteins were precipitated at −20°C overnight, followed by centrifugation at 17,000 relative centrifugal force (rcf) for 30 min. The pellet was dissolved in 0.5 ml of water, dialysed against water and filter sterilized. Dose–response experiments were performed to determine the optimal concentration of the cell-free supernatants in cell proliferation assays.

Superantigen genotyping

DNA was isolated from the AP1 strain as described (Jasir et al., 2001). Bacteria were then genotyped for their SAg profile using multiplex PCR with eight specific primer pairs for the streptococcal superantigen (ssa) gene and the pyrogenic exotoxins genes speA, speB, speC, speF, speG, speH and speJ as described in detail (Schmitz et al., 2003), and a single PCR for the streptococcal mitogenic exotoxin (sme) Z gene, using primers covering its allelic variations (5′-CAGATAGTAAATTGATTTA and 3′-AGCTAGAACGAGAATAT).

Cell lines and isolation of PBMC and T lymphocytes

BLS is a HLA class II-negative cell line generated by EBV transformation of lymphocytes from a patient with Bare Lymphocyte Syndrom (BLS) (Hume et al., 1989). BLS cells, untransfected or transfected with the HLA class II alleles DQA1*0303/DQB1*0302 (BLS-DQ3.2) or DRB1*0101/DRB1*0401 (BLS-DR4) (kindly provided by Dr G. Nepom, Seattle, USA), were maintained in RPMI 1640 medium supplemented with 10% (v/v) FBS, 100 U ml⁻¹ Penicillin and 100 mg ml⁻¹ Streptomycin (Gibco, Paisley, UK).

PBMC were isolated from heparinized blood collected from healthy volunteers. The blood was diluted 1:1 in PBS (Gibco, Paisley, UK), layered on Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) and centrifuged at 1000 rcf for 20 min at room temperature. The PBMC layer was collected and washed twice in RPMI 1640 medium supplemented with 5–10% FBS and Pen/Strep.

Human T lymphocytes were isolated from PBMC using Dynal T Cell Negative Isolation Kit (Dynal Biotech ASA, Oslo, Norway) according to the protocol provided by the manufacturer. The purity of the T cell preparation was confirmed by the lack of proliferation in response to PHA-L (1 µg ml⁻¹, Sigma, St Louis, MO).

Cell proliferation assays

PBMC were seeded in 96-well plates at a density of 1 × 10⁶ cells ml⁻¹ in RPMI 1640 medium supplemented with 5% FBS.

Streptococcal M1 protein induces T cell activation

Cells were stimulated with varying concentrations of M1 protein or bacterial supernatants (1:50–1:200 dilution) in the absence or presence of antibodies for 72 h at 37°C in 5% CO₂ and 95% humidity. Alternatively, cells were pre-treated with the M1 protein fragment A–S (1 µg ml⁻¹, final concentration) for 1 h at 37°C before stimuli were added. The cells were pulsed with 1 µCi of ³H-thymidine (specific activity 6.7 Ci mmol⁻¹; GE Healthcare, Buckinghamshire, UK) for the last 6 h of incubation. All samples were assayed in triplicates and the data presented as mean counts per minute of ³H-thymidine uptake ± SD.

To assess HLA class II dependency in these responses, untransfected and transfected BLS cells were cultured together with purified T cells, as previously described in detail (Norrby-Teglund et al., 2002). Briefly, BLS cells were treated with mitomycin C (Sigma-Aldrich, St Louis, MO) (25 µg ml⁻¹, final concentration) in order to induce mitotic arrest. Inactive BLS cells (5 × 10⁴ cells per well) were then mixed with freshly isolated human T lymphocytes (1 × 10⁶ cells per well) in 0.2 ml of RPMI medium supplemented with 5% FCS, and stimulated with M1 protein 1 µg ml⁻¹ or streptococcal supernatant diluted 1:100. PHA (1 µg ml⁻¹) served as a positive control of T cell proliferation. Cells were incubated at 37°C for 72 h, and cell proliferation was assessed by ³H-thymidine incorporation as described above.

Purification of anti-M1 protein antibodies from IVIG

A polyspecific immunoglobulin (IVIG) preparation Gamimune N (Bayer), previously reported to contain antibodies against the M1 protein (Basma et al., 1998), was used as a source for human anti-M1 protein antibodies. Recombinant M1 protein was coupled to CNBr-activated Sepharose 4B (Pharmacia Biotech, Sollentuna, Sweden) according to the manufacturer’s instructions, followed by incubation with IVIG (5 mg ml⁻¹) for 24 h at 4°C. Unbound proteins were subsequently washed away with PBS, pH 7.4, followed by washing in 50 mM sodium citrate, 0.15 M NaCl, pH 5.5 in order to remove unspecific IgG binding. Bound antibodies were then eluted with 0.5 M glycine, 0.15 M NaCl, pH 2.3 and dialysed against PBS.

T cell receptor Vβ analysis

Human PBMC (1 × 10⁶ cells ml⁻¹ in RPMI medium supplemented with 10% FBS and Pen/Strep) were stimulated with M1 protein (1 µg ml⁻¹) or PHA (1 µg ml⁻¹) for 72 h at 37°C. Cells were then washed twice in PBS containing 2% (w/v) bovine serum albumin (BSA) and stained with fluorescently labelled antibodies against different Vβ subclasses (BD Biosciences Pharmingen, San Diego, USA) and the T cell marker CD3 (anti-CD3-PE from Beckman Coulter, Marseille, France) for 20 min at room temperature. Cells were washed twice in PBS + 2% (w/v) BSA, re-suspended in 0.5 ml of PBS with 0.05% formaldehyde, and analysed in a FACSCalibur Flow Cytometer. T lymphocytes were gated based on FSC/SSC characteristics and CD3 expression.

Analysis of cytokine production at the single cell level

Human PBMC at 1 × 10⁶ cells ml⁻¹ in RPMI 1640 medium supplemented with 5% FBS were treated with M1 protein 1 µg ml⁻¹ or...
AP1 Sup diluted 1:50 in the absence or presence of IVIG 2.5 mg ml⁻¹. After incubation at 37°C for 72 h, cells were washed twice in balanced salt solution (BSS; 0.048% (w/v) CaCl₂, 0.03% (w/v) MgCl₂, 0.075% (w/v) KCl, 0.39% (w/v) Na acetate, 0.64% (w/v) NaCl and 0.17% (w/v) Na citrate), applied to individual slots on adhesion glass slides (Erie Scientific, Portsmoth, NH) and allowed to adhere for 10 min at room temperature. Bound cells were fixed in 2% formaldehyde in PBS for 15 min at room temperature. Staining for intracellular cytokines was achieved by permeabilization of the cell membranes with 0.1% saponin temperature. Staining for intracellular cytokines was achieved by permeabilization of the cell membranes with 0.1% saponin (Sigma, St Louis, MO) and immunohistochemical staining as detailed elsewhere (Norrby-Teglund et al., 1996a). The following anticytokine antibodies were used at a concentration of 2 µg ml⁻¹: anti-TNFα (LTX-21, Biosource, Camarillo, CA) and anti-IFNγ (1-D1K; MabTech, Stockholm, Sweden). Secondary biotinylated antibody goat anti-murine IgG (Caltag Laboratory, San Francisco, CA) was used at a dilution of 1:300.

**Statistical evaluation**

Data were analysed by GraphPad Prism version 4.0 for Windows (GraphPad software, San Diego, CA). Comparison between groups was performed using either the Mann–Whitney U-test or the Kruskal–Wallis median test combined with Dunn’s multiple comparison test. For paired samples, Wilcoxon signed rank test was used. P-values of less than 0.05 were considered significant.

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