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The role of myeloid receptors on murine plasmacytoid dendritic cells in induction of type I interferon

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ABSTRACT

This study tested the hypothesis that a set of predominantly myeloid restricted receptors (F4/80, CD36, Dectin-1, CD200 receptor and mannan binding lectins) and the broadly expressed CD200 played a role in a key function of plasmacytoid DC (pDC), virally induced type I interferon (IFN) production. The Dectin-1 ligands zymosan, glucan phosphate and the anti-Dectin-1 monoclonal antibody (mAb) 2A11 had no effect on influenza virus induced IFNα/β production by murine splenic pDC. However, mannan, a broad blocking reagent against mannosse specific receptors, inhibited IFNα/β production by pDC in response to inactivated influenza virus. Moreover, viral glycoproteins (influenza virus haemagglutinin and HIV-1 gp120) stimulated IFNα/β production by splenocytes in a mannan-inhibitable manner, implicating the function of a lectin in glycoprotein induced IFN production. Lastly, the effect of CD200 on IFN induction was investigated. CD200 knock-out macrophages produced more IFNα than wild-type macrophages in response to poly(I:C), a MyD88-independent stimulus, consistent with CD200's known inhibitory effect on myeloid cells. In contrast, blocking CD200 with an anti-CD200 mAb resulted in reduced IFNα production by pDC-containing splenocytes in response to CpG and influenza virus (MyD88-dependent stimuli). This suggests there could be a differential effect of CD200 on MyD88 dependent and independent IFN induction pathways in pDC and macrophages. This study supports the hypothesis that a mannan-inhibitable lectin and CD200 are involved in virally induced type I IFN induction.

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1. Introduction

Plasmacytoid dendritic cells (pDC), in contrast to conventional dendritic cells, are a rare leukocyte population which can rapidly secrete large amounts of type I IFN (IFNα/β) in response to viruses, including influenza virus, and some Toll-like receptor (TLR) ligands, e.g. CpG oligodeoxynucleotide (ODN) [1]. Although the original identification of pDC was complicated by their apparent lack of expression of surface markers associated with lymphoid and myeloid lineages e.g. CD19, CD3, CD11b, CD11c (in humans), CD13, CD33 and CD14 [2], pDC have subsequently been found to possess both lymphoid and myeloid characteristics. Murine pDC are CD11c⁺ B220⁺ CD11b⁺ Ly6C⁺ cells [1], and can also be defined using single mAbs, for example 120G8 [3] and anti-Siglec-H [4,5].

Innate immune cells express many pattern recognition receptors (PRR) that allow sensing of foreign moieties, such as TLR, C-type lectins, scavenger receptors, retinoic acid inducible gene (RIG)-I-like and Nod-like receptors. Generally, these recognize microbe-associated molecular patterns (MAMP) that are not present on host molecules, allowing discrimination between self and non-self. As viruses replicate in host cells they are composed of nucleic acids, lipids and glycoproteins derived from self molecules which makes the mechanisms of viral detection more subtle. pDCs detect viruses through TLR7 and TLR9 recognition of viral genomes (e.g. single stranded RNA and CpG containing DNA). However, viruses contain additional potential MAMP, such as glycoproteins, which may be distinguished from self through the virally encoded amino acid composition of the glycoproteins or host derived modifications. For example viral glycoproteins are presented in a repetitive array on the virus particle, the glycosylation may differ due to the cellular stress during viral infection and the properties of the glycoproteins themselves may lend themselves to different glycosylation patterns compared to host proteins. Several TLR, C-type lectins (e.g. mannose receptor, DC-SIGN, DC-SIGNR and SIGNR1), scavenger receptors and collectins bind to glycoproteins from a range of DNA and RNA viruses (reviewed in [6]). Therefore, we investigated whether the PRR on pDC were involved in viral particle and glycoprotein recognition leading to type I IFN production.

Immune cells also express immuno-modulatory receptors. Several mechanisms dampen IFNα/β production, for example, deubiquitinat-

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of multiple induction pathways [7]. CD200 and CD200 receptor (CD200R) are an immunomodulatory receptor–ligand pair which negatively regulate a range of myeloid cell functions, including proinflammatory cytokine secretion in response to lipopolysaccharide (LPS) stimulation [8]. We therefore tested whether CD200 and CD200R affected different pathways of IFNα induction.

2. Materials and methods

2.1. Mice

Cells were isolated from the 129/SvEv mouse strain unless stated otherwise. In addition, cells were prepared from CD200 knock-out (KO) [9] and C57BL/6J strains. All animals were kept and handled in accordance with institutional guidelines.

2.2. Preparation of primary cells

Single cell suspensions of splenocytes and bone marrow-derived macrophages were isolated and prepared as described previously [10–12].

2.3. Spleen cell and macrophage stimulation

Spleen cells or bone marrow-derived macrophages were stimulated in 96, 24 or 6 well tissue culture plates for 6–24 h with inactivated influenza virus, soluble glycoproteins or TLR agonists under the specific conditions indicated in each figure. Spleen cells were at 2.7×10⁷ cells/ml and macrophages at 1×10⁶ cells/ml unless otherwise stated. Where the IFN in samples was measured by enzyme-linked immunoabsorbent assay (ELISA) or bioassay, supernatants were collected and stored at −20 °C before analysis. Where IFNα/β production was measured by intracellular staining, after 5 h 1 μl GolgiPlug (GE Healthcare, Amersham, Buckinghamshire, UK) was added per ml of media and the incubation continued. At the end of the stimulation cells were detached from the plate by scraping, washed three times in phosphate buffered saline (PBS), fixed in 1% formaldehyde and stained with mAb to measure IFNα/β production by intracellular staining.

2.4. Stimuli

β-propiolactone inactivated influenza virus (A/Guangdong/25/93) (H3N2), grown in embryonated hens’ eggs and subsequently purified, was provided by M. Hocart. This virus is referred to here as inactivated influenza virus, soluble glycoproteins or TLR agonists according to the relevant figure legend.

2.5. Blocking experiments

2.7×10⁷ spleen cells/ml (unless stated otherwise in figure legend) were pre-incubated in 0.75 ml RPMI with serum for 30 min at 37 °C without any additional factors, or with the following (at concentrations indicated in the figure legends, evidence for blocking function of antibody/compound referenced where relevant): F4/80 [17], anti-Dectin-1 (2A11) [18], anti-CD36 (MF3) [19], anti-CD200 (OX90), rat IgG1 (Serotec, Kidlington, Oxfordshire, UK), rat IgG2a (Serotec) or rat IgG2b (Serotec) mAb, maleylated-bovine serum albumin (BSA) (gift from C. Neyen, University of Oxford, UK), BSA (Sigma-Aldrich, Gillingham, Dorset, UK), fucoidan (Sigma-Aldrich) [20], laminarin (from Laminaria digitata) (Sigma-Aldrich), glucan phosphate (from Saccharomyces cerevisiae) [21] (gift from D. Williams, East Tennessee State University, USA) [18,21] or mannann (from Saccharomyces cerevisiae) (Sigma-Aldrich). Cells were then stimulated with media alone, 200 or 530 haemagglutinating units (HAU)/ml inactivated influenza virus, 1 μM CpG ODN 2216, 1.3 μg/ml H1 HA, or 0.13 μg/ml gp120CNS41 (VA), with the blocking reagent still present, as described in the relevant figure legends.

2.6. Intracellular cytokine staining for IFNα and IFNβ production by pDCs

Cells were first fixed in 1% formaldehyde and then 0.2% saponin (Sigma-Aldrich) was included in all subsequent blocking, staining and wash steps. Following fixation, Fc receptors were blocked with FACS wash (0.5% BSA, Sigma-Aldrich), 5 mM EDTA, 2 mM NaN₃ in PBS supplemented with 5% rabbit serum (Invitrogen) and 4 μg/ml 2.4G2 anti-CD32/CD16 mAb [22]. Spleen cells were stained with 120G8 [3], to identify pDC, and with mAb to measure IFNα and IFNβ production (clones 4E1A and 7FD3 respectively [23], as described elsewhere [12].

2.7. FACS analysis

Data were acquired on the FACSCalibur system (Becton Dickinson, Oxford, UK) and analyzed using FlowJo software (Tree Star Incorporated, Ashland, Oregon, USA). To calculate the percentage of IFNα⁺ or IFNβ⁺ pDCs, debris was gated out by forward and side scatter characteristics and 120G8⁺ cells were selected by comparison to control staining. A gate was set where typically 5 or 10% of pDCs (120G8⁺ cells) fell within the IFNα⁺ or IFNβ⁺ gates from a sample of spleen cells stimulated with media alone. This gate was applied to all other samples to measure the percentage of IFNα⁺ or IFNβ⁺ pDCs following stimulation.

2.8. IFNα ELISA

The IFNα ELISA used is described elsewhere [12]. Briefly, plates were coated with anti-IFNα mAb (clone 4E1A) overnight and then samples were added for 2 h. IFNα was detected with a rabbit polyclonal Ab against IFNα and an alkaline phosphatase conjugated secondary and absorbance at 405 nm was recorded. To estimate the amount of IFNα in the samples, the mean absorbance for duplicate samples was compared to that from a standard curve of the IFNαA.
2.9. IFN bioassay

This assay measured IFN by the ability to inhibit Cocal virus induced cytopathic effect on L929 cells [24]. Monolayers of L929 cells in 96 well tissue culture plates were incubated overnight at 37 °C with 2-fold serial dilutions of either IFNα (from 250 U/ml) or samples in 100 μl serum free RPMI in duplicate wells. Subsequently cells were challenged with 250–2150 pfu/ml Cocal virus (gift from W. James, University of Oxford, UK) diluted in 100 μl serum free RPMI. As controls, cells were incubated without IFN in 100 μl serum free RPMI alone (cell control) or with Cocal virus only (virus control). After two days the point of 50% cytopathic effect was determined microscopically. The amount of IFN in the samples was estimated by comparison with the standard curve from the IFNαA.

![Glycoproteins stimulate IFN production in a mannan-inhibitable manner.](image)

**Fig. 1.** Glycoproteins stimulate IFN production in a mannan-inhibitable manner. Spleen cells were pre-incubated with media alone (white bars) or with (A) 5 mg/ml or (B) 0.1–5 mg/ml mannan (black bars). Cells were then stimulated with 200 HAU/ml inactivated influenza virus or in media for 6 h. IFNα and IFNβ production by pDC was measured by intracellular staining (A; n = 3, B; n = 1). (C) Spleen cells were pre-incubated with (black bars) or without (white bars) 5 mg/ml mannan and then incubated for 23 h, with 530 HAU/ml inactivated influenza virus or media (n = 2). (D) Spleen cells were stimulated with 1.3 μg/ml H1 HA (n = 7), H5 HA (n = 6), gp120 cans (n = 2), SARS spike protein (n = 1) or incubated in media alone. (E) Fc-tagged H1 HA, H5 HA and gp120 cans were incubated with GammaBind Plus Sepharose to deplete Fc tagged proteins. Volumes equivalent to 1 μg of H1 HA, H5 HA, gp120 cans and the corresponding preparations “depleted” of these Fc tagged proteins were separated by SDS-PAGE and protein was detected by silver staining (inset). In addition, spleen cells were stimulated with 1.3 μg/ml H1 HA, 1.3 μg/ml H5 HA, 0.13 μg/ml gp120 cans or equivalent volumes of the corresponding “depleted” preparations or in media alone for 23 h (n = 2). (F) Spleen cells were pre-incubated with (black bars) or without (white bars) 5 mg/ml mannan and then stimulated with HA (n = 2) or gp120 cans (VA) (n = 1) or incubated without further stimulation (media) for 23 h. In (C–F) IFN production was measured by bioassay.
2.10. Statistical analysis

As shown on each figure, n indicates the number of experiments using cells from different mice. Where 3 or more replicate experiments were conducted a 2-tailed t test or ANOVA with Bonferroni’s multiple comparison test, paired when appropriate, was performed using GraphPad Prism 4.03 software. Means were considered significantly different when p<0.05. Error bars represent the standard error of the mean.

3. Results

3.1. Lectin recognition of influenza glycoproteins leading to IFN induction

We have recently shown that murine splenic pDC from 129/SvEv mice express a range of myeloid antigens, including the predominately myeloid restricted receptors F4/80, CD36, Dectin-1, Dectin-2, mannose receptor and CD200R, and the broadly expressed CD200 [12]. Here, we investigated whether these receptors were involved in a key function of pDC, virally induced IFNα/β production. To investigate a role for a lectin in sensing influenza virus, we used mannan, a polymer of mannose, which blocks several lectins including Dectin-2, mannose receptor and SIGNR1. Treatment of spleen cells with mannan inhibited influenza virus stimulated IFNα and IFNβ production by pDC (Fig. 1A). The inhibitory effect of mannan on inactivated influenza virus-induced IFNα/β production by pDC was dose-dependent (Fig. 1B). In addition, treatment of spleen cells with mannan had little effect on cell viability (data not shown). Furthermore, mannan treatment reduced the production of functional IFNα (measured by bioassay) by spleen cells following inactivated influenza virus stimulation (Fig. 1C). This led us to hypothesize that influenza virus may induce IFNα/β production through the direct recognition of mannosylated ligands on the influenza virus particle, for example envelope glycoproteins, through a lectin.

In support of the hypothesis, a range of purified insect expressed glycoproteins (H1 and H5 subtypes of influenza virus HA, HIV-1 gp120, and SARS spike protein) were found to induce IFN production by splenocytes (Fig. 1D). The influenza virus HA and gp120 used in Fig. 1D were Fc tagged, however the Fc tag was not required for IFN induction as gp120 bearing an Fc tag mutated in residues critical for Fc receptor binding (gp120c(N54)(VA)) (Fig. 1F) as well as glycoproteins lacking an Fc tag altogether such polyhistidine tagged gp120 (data not shown) and SARS spike (Fig. 1D) all induced IFN production by spleen cells. To confirm that the IFN induction was specifically due to the HA or gp120 glycoproteins, and not a potential contaminant within the samples, these Fc tagged proteins were depleted from the preparations by incubation with protein G coated sepharose beads (GammaBind Plus Sepharose). The mock PBS treated and GammaBind Plus Sepharose treated “depleted” glycoprotein samples were separated by SDS-PAGE and proteins visualized by silver staining to demonstrate the effectiveness of the depletion by the loss of a protein band at the expected molecular weight for the glycoproteins (Fig. 1E, inset). Depletion of the HA or gp120c(N54) abrogated IFN induction (Fig. 1E). Samples were treated with polymyxin B to further confirm that the IFN induction by HA was not due to LPS. Polymyxin B treatment did not reduce IFN induction (data not shown). These data show that a range of soluble recombinant insect-expressed viral glycoproteins stimulated IFN production by spleen cells.

To investigate whether a lectin could be involved in the glycoprotein mediated IFN induction, spleen cells were treated with mannan before and during stimulation with the glycoproteins. Mannan also reduced IFN induction by H1 HA and gp120c(N54)(VA) (Fig. 1F). Trypan blue staining of spleen cells treated with mannan alone or together with the H1 HA and gp120c(N54)(VA) glycoproteins or inactivated influenza virus confirmed that mannan treatment did not affect cell viability (Supplementary Fig. 1 and data not shown). Dectin-2 is a lectin receptor expressed on pDC, which is mannann-inhibitable. Given the increasing understanding of its roles in signaling and DC activation [25], we investigated whether treatment of splenocytes with the anti-Dectin-2 blocking mAb 11E4 had any effect on inactivated influenza virus and CpG stimulated IFNα production. However we observed that 11E4 treatment had no effect on IFNα secretion (data not shown). In summary, these data suggest the involvement of a lectin in viral glycoprotein sensing, leading to IFNα/β production.

3.2. No involvement of Dectin-1 in IFNα/β induction

To assess the role of Dectin-1 in IFNα/β production by pDC, spleen cells were treated with the blocking mAb 2A11 [18] before and during stimulation with inactivated influenza virus. Treatment with this anti-Dectin-1 mAb had no significant effect on inactivated influenza virus stimulated IFNα or IFNβ production by pDC compared to treatment with an isotype control mAb or no mAb (Fig. 2A). In addition, blocking with the Dectin-1 ligands laminarin or glucon phosphatid did not affect IFNα/β production by pDC following inactivated influenza virus stimulation (Fig. 2B and C). These results suggest that Dectin-1 is not required for inactivated influenza virus stimulated IFNα/β production by pDC.

3.3. No involvement of CD36 and other scavenger receptors in IFNα/β induction

pDC express functional scavenger receptor(s) including CD36 [12,26,27], therefore we tested whether a mAb against CD36 or more general scavenger receptor ligands affected inactivated influenza virus induced IFNα/β production by pDC. Spleen cells were treated with the MF3 mAb against CD36, which has been recently found to inhibit macrophage fusion by blocking CD36 [19], before and during stimulation with inactivated influenza virus. Treatment with this anti-CD36 mAb did not significantly affect IFNα or IFNβ production by influenza virus stimulated pDC compared to untreated cells (Fig. 3A). Furthermore, incubation with fucoidan or maleylated BSA, which block CD36 and other scavenger receptors, did not significantly alter IFNα or IFNβ production by pDC compared to controls (Fig. 3B). Therefore, although pDC express CD36 we found no evidence that CD36, nor scavenger receptors more generally, have a role in inactivated influenza virus induced IFNα/β production by pDC.

3.4. F4/80 does not block IFNα/β induction

As several antibodies against highly expressed antigens on pDC alter IFNα production [4,28] and pDC clearly express F4/80 [12], we tested whether treatment of spleen cells with the mAb F4/80 altered IFNα production by pDC. The F4/80 mAb has been shown to block IFNγ release from NK cells [17], however treatment of spleen cells with F4/80 did not alter IFNα or IFNβ production by pDC (Fig. 4). Therefore, despite clear expression on pDC, no evidence was found that F4/80 plays a role in influenza virus induced IFNα/β production by pDC.

3.5. Modulation of IFNα production by CD200

As pDC and other splenocytes express both CD200 and CD200R [12], we tested whether this immunomodulatory receptor–ligand pair affected IFNα production. CD200 is known to have inhibitory effects on myeloid cells, and consistent with this we found that CD200 KO macrophages produced more IFNα than the C57BL/6J control macrophages following polyI:C stimulation (Fig. 5A). To test the effect of CD200 on pDC function, spleen cells were incubated with the OX90 mAb against CD200, which blocks CD200 interaction with
CD200R (unpublished observation, N. Barclay), or an isotype control mAb or no Ab and then stimulated with either of the MyD88-dependent stimuli CpG ODN or inactivated influenza virus. OX90 treatment significantly inhibited IFNα production by spleen cells at 23 h in response to CpG ODN stimulation (Fig. 5B). The same level of blocking was observed with OX90 concentrations as low as 10 μg/ml (data not shown). In addition, OX90 also slightly reduced IFNα production by spleen cells in response to inactivated influenza virus, however, the effect was not significant by paired ANOVA (Fig. 5C). Intriguingly, OX90 did not affect IFNα/β production by pDC following 9 or 12 h stimulation with CpG ODN (data not shown), suggesting the effects of OX90 develop over time. OX90 was not toxic as treatment of spleen cells for 23 h with 10 or 100 μg/ml OX90 or an isotype control mAb, in the presence or absence of CpG ODN, had no effect on the percentage of dead cells (data not shown). As a further control, a non-blocking mAb against CD200R OX110, was tested. This mAb, binds to CD200R, but does not disrupt its interaction with CD200 (unpublished observation, N. Barclay). OX110 had no effect on IFNα production in response to influenza virus or CpG ODN, compared to the isotype control mAb (Fig. 5D), showing that a blocking Ab against the CD200-CD200R interaction is required to inhibit the response. IFN production by CpG ODN or inactivated influenza virus stimulated C57BL/6/j or CD200 KO splenocytes was also measured by bioassay, however due to a large variation in the levels of IFN produced, this assay was not powerful enough to enable meaningful interpretation. These data suggest that CD200 promotes MyD88-dependent IFNα production, in contrast to the known inhibitory effect of CD200 on myeloid cells and the observation that CD200 deficient macrophages produced higher levels of IFNα in response to a MyD88-independent stimulus.

4. Discussion

In this study we tested whether several myeloid receptors found on pDC have a functional role in viral recognition leading to IFNα/β production. Although pDC expressed low levels of Dectin-1, no evidence was found for a Dectin-1 function in induction of IFNα/β in response to inactivated influenza virus. However, mannan, which blocks receptors recognizing mannosylated ligands including the mannose receptor, SIGN1 and Dectin-2, inhibited IFNα/β production by pDC following influenza virus stimulation, suggesting a function for such lectins in viral recognition. Studies designed to identify a function for a specific mannose binding receptor, utilizing mannose receptor KO spleen cells or the blocking anti-SIGNR1 mAb ERTR9, were inconclusive (data not shown) due to large ranges in IFN production from C57BL/6/j splenocytes and highly variable affects of ERTR9. Others have shown that several monosaccharides, mannan and anti-mannose receptor antiserum reduced IFNα/β production by human peripheral blood mononuclear cells in response to herpes simplex virus-1 [29]. Furthermore, mannan inhibited influenza virus stimulated IFNα/β production by spleen cells [30]. Together these studies implicate a mannose specific receptor and mannose receptor in particular as being involved in IFNα induction in response to several viruses either as an uptake receptor or a signaling pattern recognition receptor.
As mannan blocks receptors which recognize certain mannose-containing carbohydrates, we hypothesized that viral glycoproteins were the stimulus for the mannan inhibitable component of IFN induction. We found that highly mannosylated viral glycoproteins produced in insect cells could stimulate IFN production by spleen cells in a mannan-inhibited manner, suggesting a lectin could recognize glycosylation of the viral proteins. Further evidence for viral glycoproteins inducing IFN production is reviewed in [6]. Whether this ability to induce interferon is a property of the viral glycoprotein per se, or a result of protein expression in insect cells is currently unclear. However, recognition of glycans synthesized in insect cells is also relevant for the understanding of immune responses to insect vector-borne viruses and vaccines which use recombinant proteins expressed in insect cells.

Despite CD36 being highly expressed on pDCs we found no evidence, through use of a blocking anti-CD36 mAb or scavenger receptor ligands, that CD36 or another scavenger receptor was involved in influenza virus induced IFNα/β production by pDC. It may be interesting in future to test the requirement of CD36 in CpG induced IFNα/β production by pDC as some scavenger receptors, which are endocytic, can enable the uptake of CpG ODN which signals through TLR9 in the endosomes of pDC to induce IFNα/β production [31].

pDC also express F4/80 however the F4/80 mAb had no effect on inactivated influenza virus induced IFNα/β production by pDC. Because many antibodies against highly expressed antigens on pDC have subsequently been found to modulate IFNα production by these cells [4,28], F4/80 may be a useful tool to isolate or study pDC without interfering with pDC function.

Previous work has shown that pDC expressed clearly detectable CD200 and CD200R [12]. CD200 and CD200R are an immunomodulatory receptor–ligand pair which inhibit a range of myeloid cell functions including cytokine secretion by macrophages. MyD88-dependent TLRs recognize viruses and induce IFN in pDC, but IFN
production in macrophages is dependent primarily on a TRIF-dependent pathway (TLR-3). Because these two cell types use either MyD88- or TRIF-dependent IFN production we compared them to investigate the influence of CD200 on both MyD88 and TRIF mediated signaling pathways. CD200 KO macrophages produced more IFNα than the C57BL/6j macrophages, consistent with an inhibitory effect of CD200 in these cells. Intriguingly, the OX90 blocking mAb against CD200, significantly inhibited CpG ODN (TLR9- and MyD88-dependent) IFNα production by splenocytes and reduced inactivated influenza virus (TLR7- and MyD88-dependent) induced IFNα, suggesting that CD200 normally enhances IFNα production via MyD88-dependent pathways in these cells. It has been reported that treatment of pDC with soluble CD200 can either induce IFNα production or enhance CpG induced IFNα production [32,33], supporting the hypothesis that CD200 has positive regulatory functions. The effects of CD200 on pDC are likely to be through its receptor CD200R as CD200 itself has no known signaling pathways. CD200 differentially modulates IFNα induction pathways and have both negative and positive regulatory functions. Our results suggest that CD200 inhibits MyD88-independent IFNα induction in macrophages, while in contrast, CD200 is a positive regulator of TLR9 (Cpg ODN) and possibly of TLR7 (inactivated influenza) MyD88-dependent IFNα inducing pathways.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.intimp.2011.01.013.

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