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Evidence for a DC-specific inhibitory mechanism that depends on MyD88 and SIGIRR

Stefan K. Drexler^{†,*,}, Jeremy Wales^{*,†}, Evangelos Andreakos[†], Philip Kong[†], Alison Davis[†], Cecilia Garlanda[§], Alberto Mantovani^{§,||}, Tracy Hussell[#], Marc Feldmann[†], and Brian M.J. Foxwell^{†,&}**

[†] Kennedy Institute of Rheumatology Division, Faculty of Medicine, Imperial College London, 1 Aspenlea Road, Hammersmith, London W6 8LH, UK

[‡] Biomedical Research Foundation, Academy of Athens, Center for Immunology and Transplantation, 4 Soranou Ephessiou, 115 27 Athens, Greece

[§] Istituto Clinico Humanitas and Fondazione Humanitas per la Ricerca, I-20089 Rozzano, Italy

^{||} University of Milan, 20126 Milan, Italy

[#] Imperial College London, National Heart and Lung Institute, Sir Alexander Fleming Building, Exhibition Road, South Kensington, London, SW7 2AZ, UK

Abstract

Dendritic cells (DCs) are an essential link between the innate and adaptive immune response. In order to become effective antigen presenting cells DCs need to undergo maturation, during which they up-regulate co-stimulatory molecules and produce cytokines. There is great interest in utilising DCs in vaccination regimes. Over recent years, Toll-like receptor (TLR) signalling has been recognised to be one of the major inducers of DC maturation. This study describes a mutant version of the TLR adaptor molecule MyD88 (termed MyD88lpr) as a novel adjuvant for vaccination regimes. MyD88lpr specifically activates DCs by disrupting a DC intrinsic inhibitory mechanism, which is dependent on SIGIRR. Moreover, MyD88lpr was able to induce an IgG2a dominated response to a co-expressed antigen, suggesting Th1 immunity. However, when used as a vaccine adjuvant for Influenza Nucleoprotein there was no significant difference in the lung viral titres during the infection. This study describes MyD88lpr as a potential adjuvant for vaccinations, which would be able to target DCs specifically.

Keywords

Toll-like receptors; SIGIRR; inflammation

Introduction

In response to an infection the affected tissue initiates a defensive response consisting of an immediate innate immune response, followed by the induction of highly specific adaptive

Correspondence: Marc Feldmann, Kennedy Institute of Rheumatology Division, Faculty of Medicine, Imperial College London, 1 Aspenlea Road, Hammersmith, London W6 8LH, UK, phone no. +44(0) 20 8383 4444, m.feldmann@imperial.ac.uk.

&Deceased

* S. K. Drexler and J. Wales contributed equally to this study.

** Current Address: Department of Biochemistry, University of Lausanne, Chemin des Boveresses 155, 1066 Epalinges, Switzerland

Conflict of interest

The authors declare no conflict of interest

immunity. Dendritic cells (DCs) are essential for detecting invading pathogens and initiating as well as regulating the adaptive immune response.

As key initiators of immunity, DCs are the target of many novel vaccine regimens. *Ex vivo* production and antigen exposure of DCs are commonly utilised as an approach to enhance immune responses against cancers [1,2]. However there are methodological draw-backs of this individualised therapeutic approach. Other molecules have been included as part of the vaccine to target DCs *in vivo* such as DEC205 (Dendritic and epithelial cell 205). However, this approach required adjuvant-like molecules (anti-CD40), as well as the DEC205, to act as a specific adjuvant [3]. More recently, intracellular signalling molecules, such as those of the TLR (Toll-like receptor) pathway, have been assessed *in vivo* as a novel group of adjuvants by over-expressing them as part of vaccine constructs [4]. Wild-type MyD88 (Myeloid differentiation primary response gene 88) and TRIF (TIR domain containing adaptor inducing IFN β) were expressed as part of a DNA vaccine which induced Th1 immune responses and induced some protection in an influenza model [5]. NIK (NF- κ B inducing kinase) has also been used to generate strong Th1 responses with a specific CTL (Cytotoxic T lymphocyte) response [6]. And yet, these approaches do not specifically target the DC.

In recent years, TLR signalling pathways have been described to be one of the major activators of DCs by being able to recognise PAMPs (pathogen associated molecular patterns) and induce signalling pathways that ultimately lead to cytokine production and up-regulation of major histocompatibility complex (MHC) II by DCs [7]. Studies on TLR signalling, performed in gene deficient mice or cell lines, have shown that it is dependent on four intracellular adaptor molecules [7]. MyD88, the first one to be described, was shown to be ubiquitously used by all TLRs with the exception of TLR3. Mal (MyD88 adaptor like) is used by TLR2 and TLR4, TRIF by TLR3 and TLR4 and TRAM (TRIF related adaptor molecule) by TLR2 and TLR4. A fifth adaptor molecule, SARM (Sterile-alpha and armadillo motif containing protein), was shown to inhibit TRIF-dependent signalling [8]. The recruitment of these adaptor molecules is one of the earliest events after TLR stimulation and is dependent on TIR (Toll-Interleukin-1R domain) domain interactions between the receptor and the adaptor molecules. Studies in human cells using the expression of dominant negative (dn) versions of the adaptor molecules have generally agreed with the data obtained from knock-out mice. However, a notable exception was the observation that a dominant negative version of MyD88 (termed MyD88lpr) functions as an inhibitor of TLR4 signalling in human cell lines and HUVECs (human umbilical vein endothelial cells) but not in human macrophages [9,10].

In this study we investigated the function of MyD88lpr in human and murine DCs. We describe that MyD88lpr specifically activates DCs *in vitro*. This is attributable to the expression of a wild-type TIR domain and the disruption of a DC specific inhibitory mechanism, which is dependent on SIGIRR (Single immunoglobulin IL-1R related protein, also termed TIR8). Furthermore, due to its DC activating effect MyD88lpr is shown to be an effective adjuvant *in vivo*, where it was able to induce an enhanced antibody but is not sufficient to generate protective immunity against Influenza challenge.

Materials and Methods

Antigens, adjuvants and adenoviral constructs

Green fluorescent protein (GFP) was supplied by Roche (Indianapolis, USA). The X-31 strain of Influenza virus was supplied by Tracey Hussell, Imperial College London. For some experiments, virus was heat-inactivated at 56°C for 30 minutes.

Replication defective adenoviral vectors having no insert (Ad0) or encoding either GFP (AdGFP, control), MyD88lpr and GFP (AdMyD88lpr), wild-type Mal and GFP (Malwt), dominant negative Mal and GFP (Maldn), TIR domain of Mal and GFP (Mal TIR), wild-type TRAM and GFP (TRAMwt), dominant negative TRAM and GFP (TRAMdn) or TIR domain of TRIF and GFP (TRIF TIR) were constructed, propagated, purified and titered as previously described [11]. To create AdNP (Neucleoprotein) and Ad(MyD88lpr)NP the NP and MyD88lpr genes were inserted into multiple cloning site (MCS) 1 and 2 of pENTR.GD.for (Figure S1) respectively and combined with the viral genomes as per the manufacturer's instructions (Invitrogen, Paisley, UK). Viruses were then propagated, purified and titered as previously described [11]. The plasmid encoding Flag-tagged, wild-type human SIGIRR was kindly provided by X. Li (Lerner Research Institute, Cleveland, Ohio, USA). The human SIGIRRwt, MyD88wt, MyD88Pro200His and MyD88 DD constructs were cloned into MCS of pENTR4.3F vector (Figure S1) using Kpn I and Sma I restriction enzymes. Viruses were then propagated, purified and titered as previously described [11]. A description of all the TLR adaptors and their respective mutants used is found in Figure S2.

Mice and immunizations

Eight to ten week old Balb/c females were purchased from Harlan UK (Bicester, UK) and maintained under strict pathogen free conditions for the duration of the experiments. Mice were vaccinated subcutaneously with adenoviral vectors in 100µl phosphate-buffered saline (PBS) or rGFP in complete Freund's adjuvant. The dose of vaccine was either 10^7 or 10^6 infectious units of adenovirus or 20µg rGFP in adjuvant. Mice were tail-bled after injections and serum isolated for storage at -20°C for subsequent analysis by ELISA.

For Influenza challenge, mice were administered 50HA Influenza X-31 intranasally under inhalational anaesthetic. Weight was monitored daily. Influenza lung titres were assessed through Madin-Darby canine kidney (MDCK) infection as previously described [12].

These trials have been approved by the Ethical Review Process Committee and the UK Home Office.

Growth and infection of murine and human dendritic cells

Bone-marrow was harvested from 8–10 week C57BL/6 mouse femurs of a wild-type or MyD88^{-/-} background and cultured with GM-CSF (Peprotech, Rocky Hill, NJ, USA). For dendritic cells 20ng/ml GM-CSF was used with an additional 20ng/ml added at day 4. Non-adherent dendritic cells were isolated at day 6–7. Macrophages were cultured in 10ng/ml GM-CSF for 6–7 days, as described before [13]. Dendritic cells and macrophages were infected with adenoviral vectors at an M.O.I. of 300. During infection cells were spun for 1 h at 1500 rpm and 37°C before being rested for 1 h in the incubator, after which the virus was removed and replaced with fresh RPMI containing 10% foetal calf serum and growth factors.

Human monocytes were isolated from whole blood as previously described [14]. Monocytes were cultured with GM-CSF and IL-4 for 5–7 days as described before [14,15]. Non-adherent dendritic cells were isolated and infected with adenoviral vectors at an M.O.I. of 100. For macrophage differentiation monocytes were cultured in M-CSF (Peprotech, London, UK) for 3–4 days. Adherent macrophages were isolated and infected with adenoviral vectors at an M.O.I. of 100.

Rheumatoid arthritis synovial fibroblasts (RASf) were isolated from joint samples as described before [16].

ELISA

Serum IgG1 and IgG2a levels against GFP were assessed by ELISA and compared to purified mouse immunoglobulin standards (Sigma, Missouri, USA). Plates were coated with 50ng rGFP per well. Influenza NP was detected by lysing HEK293 cells in pure H₂O by freezing (−70°C) and thawing (37°C) in three cycles and then making up to a final concentration of 1× PBS. Lysates were then used to coat a 96-well ELISA plate and Nucleoprotein detected by 1µg/ml anti-Influenza NP (Antibody Shop, Gentofte, Denmark) and sheep anti-mouse HRP conjugate (Amersham Biosciences, New Jersey, USA). TNFα and IL-6 levels in cell-culture supernatants were also measured by ELISA (BD Pharmingen, California, USA).

Western blotting

Cells were lysed in 1% NP40, 20mM Tris and soluble lysates run on a 10% polyacrylamide gel (Pierce, Rockford, IL, USA). Gels were transferred to a PVDF membrane (PerkinElmer) and probed with 0.5 µg/ml Rabbit anti-MyD88 (Abcam, Cambridge, UK) and sheep anti-rabbit HRP conjugate (Amersham Biosciences, New Jersey, USA).

Immunoprecipitation

5 × 10⁶ cells per condition were treated appropriately, washed in ice-cold 1× PBS and lysed for 20 min in 1 ml NP-40 lysis on ice. Protein G sepharose beads were washed 3× in lysis buffer, and 50 µl added to each sample for pre-clearing end over end at 4°C for 30 min. Samples were spun at 4°C at 10,000 rpm for 1 min and supernatants removed into fresh pre-cooled tubes. Immunoprecipitation with 5 µg/ml specific/isotype control antibody was allowed to proceed for 1.5 h at 4°C end over end. Washed protein G beads were then added to each sample (50 µl each) and samples incubated a further hour at 4°C end over end before spinning at 10,000 rpm for 1 min at 4 °C. Supernatants were removed, and were washed 3 times with lysis buffer prior to addition of 500 mM NP-40 lysis buffer for denaturing and separation from the beads.

Analysis of the activation status of DCs

DC activation was assessed as previously described [6]. Nuclear extracts were probed for the DNA-binding of NF-κB by EMSA (electrophoretic mobility shift assay). Cell-surface expression of CD80 and CD86 was analysed by flow-cytometry. Proliferation of allogeneic human blood and GFP-specific T cells was assessed by the incorporation of tritiated-thymidine. GFP-specific T cell lines were generated as described before [17].

Statistical methods

For statistical analysis a one-way ANOVA was performed with a Student-Newman-Keuls multiple comparisons test to compare each column (GraphPad InStat v3.06, California, USA).

Results

Expression of MyD88lpr induces the spontaneous maturation of human DCs

As we previously observed that MyD88lpr (a mutation construct of MyD88 lacking parts of the death domain (DD) and with an additional mutation in the TIR domain, see figure S2) had no effect on LPS signalling in macrophages [9,18] we further investigated its effect in human DCs. Surprisingly, rather than inhibiting LPS signalling, expression of MyD88lpr resulted in a 5 fold super-induction of TNFα production in human DCs (Figure 1A). Also MyD88lpr caused the spontaneous release of TNFα from resting DCs (Figure 1B). This was not an effect of virus infection as control constructs induced no cytokine production above

background (Figure 1A and B). Similar results were obtained for IL-6 production (Figure S3).

Further investigation of this phenomenon showed that the expression of MyD88lpr resulted in the spontaneous activation of NF- κ B as observed by EMSA (Figure 1C), which explains the spontaneous production of TNF and IL-6. Furthermore, the effects of MyD88lpr were not restricted to cytokine production as the co-stimulatory molecules CD80 and CD86 were also up-regulated (Figure 1D). The spontaneous maturation of human DCs in the absence of any further stimuli, induced by MyD88lpr resulted in enhanced antigen presenting function as measured in a mixed lymphocyte reaction (Figure 1E). Furthermore, using the presence of the GFP in our adenoviral construct as a specific antigen we demonstrated that the presence of MyD88lpr enhances the antigen presentation to GFP specific T cells (Figure 1F).

Over-expression of MyD88wt has no effect on DC activation

To further investigate this unexpected observation, other MyD88 constructs were subjected to similar analysis. Neither murine nor human MyD88wt had any effect on resting or LPS stimulated human DC cytokine production (Figure 2A to D). However, a MyD88 construct containing solely the DD super-induced TNF production in LPS stimulated human DCs by 3 fold, although, the enhancing effect did not reach significance in resting human DCs (Figure 2C and D). These data suggested that interruption of MyD88 function in resting cells causes an activation of human DCs. Therefore, the function of other TLR adaptor molecules was further investigated.

Over-expression of a wild-type TIR domain is responsible for DC activation

This study examined whether the manipulation of levels of other TLR adaptor molecules would have an effect on DC function. In contrast to MyD88wt, Malwt and TRAMwt resulted in the production of TNF α from resting human DCs and enhanced the production of TNF α from LPS stimulated human DCs (Figure 2A and B). Furthermore, over-expression of just the Mal or TRIF TIR domains also resulted in the same effect (Figure 2E and F). However, the disruption of the TIR function in Mal and TRAM resulted in loss of this phenomenon in human DCs (Figure 2G and H). These results were also reflected in the production of IL-6 (Figure S4).

MyD88wt reverses activating effect of MyD88lpr

These results would suggest that the over-expression of either just a TIR domain or to a lesser extent a DD results in the enhancement of human DC activity. However, as observed with MyD88wt a TIR domain together with a DD had no effect on DC function. Therefore, we investigated whether MyD88wt would act as a competitor to the activating effect of MyD88lpr. As shown in figure 2I and J MyD88wt dose-dependently inhibited the stimulatory effect of MyD88lpr on TNF α production in both resting and LPS stimulated human DCs back to the level of control cells but not further, which was also observed in case of IL-6 production (Figure S4). This data suggested that MyD88lpr is disrupting an inhibitory function of MyD88wt.

MyD88lpr can activate murine DCs, but not macrophages, and this effect requires endogenous MyD88

So far these results were all performed in human DCs. We therefore were interested to see, whether similar phenomena are observed in murine cells. Similar to human macrophages, over-expression of MyD88wt or MyD88lpr had no effect on bone marrow derived macrophage function. However, similar to human DCs MyD88lpr causes the spontaneous production of TNF α and IL-6 by their murine counterparts (Figure 3 and Figure S5).

Given our hypothesis that MyD88lpr is disrupting an inhibitory function of endogenous MyD88 leading to DC activation we investigated whether the spontaneous DC activation by MyD88lpr can be observed in MyD88 deficient cells. As shown in Figure 3 and Figure S5 MyD88lpr was unable to induce TNF α or IL-6 production in the absence of endogenous MyD88.

SIGIRR interacts with MyD88 and is required for MyD88lpr induced DC activation

The data so far suggests that MyD88lpr or the over-expression of other TIR domain containing molecules disrupts an “inhibitory mechanism” provided by endogenous MyD88 in DCs. Furthermore, MyD88wt seems to be able to competitively reverse the activating effect of MyD88lpr (Figure 2G and H). This would suggest that MyD88 interacts with another TIR domain containing molecule that is present in DCs but not in macrophages. Previous studies have shown that the orphan receptor and IL-1R family member SIGIRR (also known as TIR8) is an inhibitor of TLR signalling and is expressed in DCs but not macrophages [19–21]. Therefore, this study investigated whether SIGIRR is the TIR domain containing molecule in question. In agreement with this hypothesis it was observed that MyD88lpr is unable to activate SIGIRR-deficient DCs, whereas reintroduction of wild-type SIGIRR together with MyD88lpr again resulted in the spontaneous induction of TNF α by those cells (Figure 4A).

Although, it has previously been shown that MyD88 interacts with SIGIRR following HEK293 cell activation [22] our studies in DCs would require that MyD88 associates with SIGIRR constitutively in immature DCs. As shown in Figure 4B this is the case. In the upper panel (Figure 4B) the precipitation of Flag-tagged SIGIRR co-immunoprecipitates HA-tagged MyD88wt. Furthermore, the introduction of a mutation in the TIR domain of MyD88 (MyD88Pro200His) reduced co-immunoprecipitation, although some interaction was still observed. There was no interaction observed between the DD of MyD88 and SIGIRR. Also, there was no precipitation observed using an isotype control antibody. The middle and lower panel of Figure 4B shows that all constructs were expressed at roughly equal levels.

MyD88lpr can act as a potential DC specific adjuvant *in vivo* and leads to increased IgG2a antibody production

Given the potent effect of MyD88lpr inducing spontaneous maturation of DCs we considered whether it can be used as an adjuvant. Since the constructs contained GFP it was used as an antigen. Mice were vaccinated subcutaneously with AdGFP in PBS, Ad(MyD88lpr)GFP in PBS or 20 μ g of rGFP in complete Freund's adjuvant. At 14 and 56 days after vaccination there was no detectable IgG1 to GFP in the serum of the adenovirally infected animals (Figure 5A). However, IgG2a was detected in the adenovirally vaccinated animals and a clear trend of increased IgG2a levels was observed in Ad(MyD88lpr)GFP vaccinated animals (Figure 5B), suggesting that MyD88lpr induces a strong antibody response which is predominantly type-1 (Th1). This is arguably the desired immune response required to control many viral infections. The adenovirally vaccinated mice were then boosted with the same constructs and 14 days later antibody responses assessed. After boosting, MyD88lpr induced an enhancement in both IgG1 and IgG2a antibody responses (Figure 5A and B), thus indicating that upon boost these regimens can induce a more balanced mix of type-1 and 2 immune responses.

MyD88lpr cannot adjuvant a protective response in the lungs during influenza challenge

Given the above data with GFP as a model antigen, this study further investigated the effect of MyD88lpr as an adjuvant in a more medically relevant model of influenza. To date influenza vaccines are based on cocktails of highly variable coating proteins of the virus. A

vaccine based on nucleoprotein would not suffer from the variation of viral strains if a reasonably potent immune response could be induced. We therefore, generated adenoviral constructs that expressed influenza nucleoprotein (NP) with or without MyD88lpr (Figure S6). These were used in the X-31 challenge model. Mice were vaccinated with 10^7 pfu of AdNP, Ad (MyD88lpr)NP or Ad0 subcutaneously, and boosted on day 28 with a lower dose of adenovirus (10^6). On day 49 mice were challenged with 50HA of live Influenza X-31 intranasally. Weight was monitored daily where no significant difference was seen in any of the groups (Figure 5C). However, the Ad (MyD88lpr)NP vaccinated animals may recover faster from infection. Figure 5D shows that mice vaccinated with AdNP or Ad (MyD88lpr)NP were able to clear viral titres from the lung by day 4, whereas those mice that received empty adenovirus were unable to clear virus from the lung until day 6. However there was no significant benefit to including MyD88lpr in the vaccine.

Discussion

Amongst cells of the innate immune response, DCs hold a special place. These cells are not only major players in the innate immune system but are professional antigen presenting cells that are the main initiators of the adaptive response. Therefore, it is essential that the maturation and activation of these cells is tightly controlled. And from a practical point of view, DCs are also the target of several strategies to boost the efficacy of vaccination protocols. However, barring the use of *ex vivo* manipulated DCs there is no approach that would target these cells specifically. In this study we have unexpectedly uncovered a control mechanism in resting DC involving the sequestration of MyD88 by the orphan IL-1R family member, SIGIRR. The disruption of this interaction results in the spontaneous activation of resting DCs as well as the potent enhancement of activated DCs, but not of other cells, such as macrophages. Given that this mechanism would be restricted to DCs we show that this phenomenon could be potentially harnessed as a DC selective vaccine adjuvant that selectively boosts the Th1/cytotoxic T cell response.

The unexpected result of MyD88lpr and other functional TIR domain containing constructs to induce DC activation led us to hypothesise that there is a DC specific, constitutive inhibitory mechanism. This would be dependent on MyD88 constitutively bound to a TIR domain containing receptor. This hypothesis proposes that the over-expression of a TIR domain would displace MyD88 from the inhibitory receptor, thereby impairing the inhibitory signal. Consequently immature DCs become activated and start producing cytokines. Over-expression of MyD88wt would have no effect since the inhibitory mechanism is dependent on MyD88. Furthermore, the co-expression of MyD88lpr together with MyD88wt reverses the activation of DCs by competing for binding to the inhibitory receptor.

The proposed inhibitory receptor has to be selectively expressed in immature DCs but not in macrophages. To our knowledge, only one TIR domain containing receptor has so far been described to have such a restricted expression pattern in mice and humans, the orphan receptor SIGIRR [19,20]. Since no activating function of SIGIRR has been described so far, it might be a candidate for the inhibitory mechanism proposed by us. Indeed, SIGIRR^{-/-} DCs become activated as a result of MyD88lpr expression only when SIGIRR is reintroduced by adenoviral infection. Furthermore, our hypothesis requires a dual function of MyD88, acting as an inhibitor as well as signal transducer. Into and colleagues recently identified a TLR inhibitory function of MyD88, which depends on S-nitrosylation of the conserved cysteine 216 in the TIR domain of MyD88 [23]. Interestingly, only two other TIR domain containing molecules contain a corresponding cysteine residue, Mal and SIGIRR [23].

The model of an inhibitory mechanism dependent on SIGIRR and mediated by MyD88 would also postulate a constitutive interaction between those proteins. A previous study has shown transient interaction between MyD88 and SIGIRR following IL-1R or TLR4 stimulation in the HEK293 cell line [22]. The study presented here shows that SIGIRR interacts with MyD88 through homotypic TIR domain interactions in unstimulated primary human DCs without further stimuli.

Based on the results of this study one might expect SIGIRR deficient DCs to become spontaneously activated. Indeed, in certain circumstances a spontaneous activation of SIGIRR deficient epithelial cells is observed [24]. Furthermore, SIGIRR deficiency is strongly associated with autoimmunity, as even heterozygous SIGIRR deficient mice die from accelerated systemic lupus erythematosus (SLE) at an early age when backcrossed to an MRL^{lpr/lpr} background [25]. The development of SLE in these mice was shown to be due to increased activation of DCs. However, other mechanism might compensate for the loss of SIGIRR and subsequently unwanted DC maturation. These include the C-type lectin receptors DC-SIGN and Dcir, which have been identified as negative regulators of DC maturation [26,27].

Similar to human DCs, MyD88lpr was able to activate DCs but not macrophages derived from murine bone-marrow, whereas MyD88wt could not. In mice, MyD88wt was shown to be a successful adjuvant when co-expressed in a DNA vaccine which partially protects against a tumour and Influenza challenge [5]. However, in this study the activation of DCs was not assessed. Our results suggest that the enhancement seen by MyD88wt would not be conferred through an activation of DCs, but through the activation of other cell types similar to our studies using NIK as an adjuvant [6].

As observed in other studies using intracellular signalling molecules, MyD88lpr was able to induce an IgG2a dominated response to a co-expressed antigen, suggesting Th1 immunity [5,6,28,29]. When MyD88lpr was used as a vaccine adjuvant for Influenza Nucleoprotein there was no significant difference in the lung viral titres during the infection when comparing AdNP or Ad(MyD88lpr)NP vaccinated animals, yet they were both significantly better at clearing the infection than animals administered empty adenovirus. The fact that both ADNP and Ad(MyD88lpr)NP did not affect weight loss in these animals would suggest that our choice of antigen was not ideal. The more common choice of antigen is Influenza haemagglutinin and/or neuraminidase to induce protective antibody. Perhaps through the use of these antigens we would see a protective response that would be further enhanced by the use of MyD88lpr. Another challenge model using a low efficiency protective antigen, such as the T and B epitopes of haemagglutinin [30], may show greater effect if linked to MyD88lpr.

In summary this study describes an inhibitory mechanism dependent on MyD88 and SIGIRR in immature DCs, which results in the maturation of DCs when disrupted by the expression of a TIR domain. The elucidation of this mechanism will potentially allow us to design vaccination strategies that target DCs and increase their efficacy in activating immune responses.

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Abbreviations used in this paper

TIR	Toll/IL-1R
SIGIRR	Single immunoglobulin IL-1R-related
DEC205	Dendritic and epithelial cell 205
TRIF	TIR domain containing adaptor inducing IFN β
NIK	NF- κ B inducing kinase
Mal	MyD88 adaptor like
TRAM	TRIF related adaptor molecule
SARM	sterile-alpha and armadillo motif containing protein

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Figure 1. MyD88lpr expression spontaneously induces human DC maturation as measured by TNFα production (A and B), NF-κB activation (C), upregulation of CD80 and CD86 (D) and T cell priming (E and F)

Human dendritic cells were used at day 5 of in-vitro differentiation with 50 ng/ml GM-CSF and 10 ng/ml IL-4 in RPMI 5% FCS. Cells were left uninfected, or infected (100:1 m.o.i.) for 2 h in serum-free medium with adenoviruses expressing GFP, GFP and murine MyD88lpr or no insert. (A and B) 24 h post infection cells were stimulated with 100 ng/ml LPS (A) or left un-stimulated (B) for a further 18 h before supernatants were collected and TNFα induction assayed by ELISA. Data are expressed as mean ± SEM. n = 4, data is n independent experiments pooled. $p \leq 0.05$ *; $p \leq 0.01$ **. (C) Cells were lysed at 6 and 24 hours and nuclear extracts obtained to detect NF-κB binding by EMSA. (D) At 48 hours cells were obtained and stained for CD80 and CD86 cell surface expression by flow cytometry. (E) After 48 hours graded doses of dendritic cells were cultured with 1×10^5 allogeneic T cells and proliferation was measured at day 6 by the incorporation of tritiated-thymidine (cpm). (F) After 48 hours graded doses of dendritic cells were cultured with 2×10^4 antigen-specific T cells and proliferation was measured at day 3 by the incorporation of tritiated-thymidine (cpm). (C–F) Experiments shown are representative of 3 independent experiments.

Figure 2. Expression of a wild-type TIR domain results in the spontaneous activation of human DCs (A to H), which can be reversed by co-expression of MyD88wt (I and J)
 Human dendritic cells were used at day 5 of in-vitro differentiation with 50 ng/ml GM-CSF and 10 ng/ml IL-4 in RPMI 5% FCS. Cells were left uninfected, or infected (100:1 m.o.i.) for 2 h in serum-free medium with adenoviruses expressing GFP (control), A and B) murine MyD88wt, Malwt or TRAMwt, C and D) human MyD88wt or human MyD88DD, E and F) Mal TIR or TRIF TIR, G and H) Maldn or TRAMdn. 24 h post infection cells were left unstimulated (A, C, E and G) or stimulated with 100 ng/ml LPS (B, D, F and H) for a further 18 h before supernatants were collected and TNFα induction assayed by ELISA. (I–J) Cells were left uninfected, or infected (100:1 m.o.i.) for 2 h in serum-free medium with adenoviruses expressing GFP (control), murine MyD88wt, MyD88lpr or combinations of the two later viruses. An m.o.i. of 100 was used for MyD88lpr or control viruses and MyD88wt was used at m.o.i. 200 or 100 as appropriate. 24 h post infection cells were stimulated with 100 ng/ml LPS for 18 h, where after supernatants were collected and TNFα induction assayed by ELISA. Data are normalised to MyD88lpr only infected cells. Data are expressed as mean ± SEM. n = 4 (A to H) or n = 3 (I and J), data is n independent experiments pooled. p ≤ 0.05 *; p ≤ 0.01 **

Figure 3. MyD88lpr can activate murine DCs, but not macrophages, and this effect requires endogenous MyD88

Murine dendritic cells (DC) and murine macrophages (MQ) were cultured from bone-marrow and cultured in GM-CSF for 6–7 days from wild-type or MyD88^{-/-} mice. Cells were left uninfected, or infected (300:1 m.o.i.) for 2 h in serum-free medium with adenoviruses expressing GFP (control), murine MyD88wt or MyD88lpr. 24 h post infection supernatants were collected and TNFα induction assayed by ELISA. Data are expressed as mean ± SEM. n = 3; data is 3 independent animals pooled. p ≤ 0.05 *; p ≤ 0.01 **.

Figure 4. SIGIRR is required for MyD88lpr mediated DC activation (A) and SIGIRR associates with MyD88 through homotypic TIR domain interactions in human immature DCs (B)

(A) Murine dendritic cells (DC) were cultured from bone-marrow and cultured in GM-CSF for 6–7 days from SIGIRR^{-/-} mice. Cells were left uninfected, or infected (300:1 m.o.i.) for 2 h in serum-free medium with adenoviruses expressing GFP (control), murine MyD88lpr or SIGIRRwt. 24 h post infection supernatants were collected and TNF α induction assayed by ELISA. Data are expressed as mean $\pm$ SEM. n = 4; data is 4 independent animals pooled. $p \leq 0.05$ *; $p \leq 0.01$ **. (B) Human DCs were used at day 5 of *in vitro* differentiation with 50 ng/ml of GM-CSF and 10 ng/ml of IL-4 in RPMI 5% FCS. Cells were left uninfected, or double infected (100:1 m.o.i.) for 2 h in serum-free medium with adenoviruses expressing SIGIRR wt and GFP (control), human MyD88 wt, human MyD88 Pro200His (point mutation of AS200 Pro to His) or human MyD88 DD (TIR domain deleted). Cells were left over-expressing for 24 h before being lysed. SIGIRR was immuno-precipitated using an anti-Flag antibody (Sigma, Poole, Dorset, UK) and MyD88 constructs were detected by western blot using an anti-HA antibody (Covance, California, USA). Experiment shown is representative of 3 independent experiments.

Figure 5. MyD88lpr can adjuvant vaccination and induce an IgG2a dominated response, but cannot adjuvant protective immunity for Influenza challenge

(A–B) Balb/c mice were administered 20 μ g rGFP in complete Freund's adjuvant (CFA), 10⁷ adenoviruses expressing GFP (AdGFP) or 10⁷ adenoviruses expressing GFP and MyD88lpr (Ad(MyD88lpr)GFP) in PBS. Adenovirally vaccinated animals were boosted at day 56 with 10⁶ of the same adenovirus primed with. All mice were bled at day 14 and 56 after prime and day 14 after boost. Serum titres of IgG1 (A) and IgG2a (B) reactive to GFP were then assessed by ELISA. Data are expressed as mean $\pm$ SEM, normalised to day 14 serum of those animals that received rGFP. Data is 5 animals assayed independently. (C–D) Balb/c mice were vaccinated with 10⁷ adenoviruses expressing Nucleoprotein (AdNP), Nucleoprotein and MyD88lpr (Ad(MyD88lpr)NP) or no gene (Ad0) in PBS. Twenty-eight days later mice were administered a further 10⁶ pfu of the same adenovirus. Twenty-one days after boost mice were challenged with 50HA Influenza X-31 intranasally. (C) Weight was monitored daily after Influenza challenge and is shown as a percentage of starting weight. (D) Influenza lung titres were assessed by quantification of infections on MDCK cells and are shown as plaque forming units (pfu) per lung. Data are expressed as mean $\pm$ SEM. Data is 3 animals assayed independently. $p \leq 0.05$ *; $p \leq 0.01$ **. Experiment shown is representative of 3 independent experiments.