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Immunisation with virion-loaded plasmacytoid or myeloid dendritic cells induces primary Th-1 immune responses

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Abstract

Dendritic cells (DCs) induce different types of immune responses depending on their lineage and activation signals. When exposed to inactivated pseudorabiesvirus (iPRV), plasmacytoid but not myeloid DCs released IFN- α and IL-12. Remarkably, both iPRV-pulsed DC types were able to induce primary IFN- γ producing T cells and IgG isotype switching *in vivo*. In contrast, tetanus toxoid pulsed DCs did not induce detectable primary immune responses. The efficacy of antiviral T and B cell priming proved dependent on the recipient's genotype. We conclude that either plasmacytoid or myeloid DCs pulsed with inactivated virus suffice to induce primary Th1-polarised immune responses. © 2004 Elsevier Ltd. All rights reserved.

Keywords: Dendritic cells; Virus; Immunisation

1. Introduction

Activation of resting T cells strictly depends on dendritic cells (DCs), which present antigenic peptides in the context of major histocompatibility complex (MHC) in the T cell areas of the draining lymph nodes. Different DC subsets, which can be distinguished based on origin, phenotype, and function [1,2], express pattern-recognition receptors, allowing them to discriminate between invading micro-organisms and self-antigens [3] and provide membrane-bound and secretory signals for T cell priming. Murine plasmacytoid DCs descending from lymphoid precursors express CD11c and CD8 α , but not CD11b and are thought to stimulate the differentiation of T helper (Th)-1 cells by the production of IL-12. Conversely, DCs of myeloid origin, which carry CD11c and CD11b, but not CD8 α , prime Th-2 responses [4,5].

Which DC subsets become activated by non-replicating virion structures, and whether these suffice to enable prim-

ing of naive T cells is largely unknown [6]. Acute viral infections typically evoke Th-1 type immune reactions, which are characterised by high interferon (IFN)- γ production essential for virus-specific IgG2a responses [7–12]. Our studies are aimed at identifying critical factors that guide the host's antiviral immune reactions. To our surprise, the Th-1 polarized immune responses, typical for acute α -herpesvirus and coronavirus infections, failed to default to a Th-2 profile despite the functional absence of p40 dependent interleukin (IL)-12 and IL-23, as well as IFN- α/β or IFN- γ [13,14], as has been tested in gene-deleted mice. Later, others noted an IL-12 independent Th-1 development in respiratory Sendai, adenovirus and influenza virus infection [8–10], and in systemic murine cytomegalovirus [11] vesicular stomatitis and lymphocytic choriomeningitis virus infections [12]. Although several reports state that type 1 interferons do play a role in the development of IFN- γ producing Th cells [15], both live and killed viruses can induce Th-1 responses in IFN- α/β -receptor deficient mice [16]. In general, the role of IFN- α/β , which is produced in high amounts upon virus infection by plasmacytoid DC precursors in Th cell polarization remains disputable.

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In recent attempts to delineate antiviral Th-1-biased responses we examined the reactions to inactivated pseudorabies virus (iPRV), a herpes simplex virus-related α -herpesvirus. Exclusion of viral replication prevents target cell lysis and allows control of the antigen load, and immunization with such preparations mimics vaccination with inactivated vaccines that are often not adjuvanted. We observed that inactivated viruses from different families, similar to authentic infections, are able to induce specific IgG2a production driven by a type 1 immune response, with dominant IFN- γ production by splenocytes [43] IgG2a production induced by iPRV was impaired only in gene-deleted mice lacking IFN- γ receptor function, but remained unaffected in IL-12p40 $^{-/-}$ or IFN- α/β R $^{-/-}$ mice. These results placed IFN- γ as a critical mediator of iPRV-induced IgG2a class switching.

The present study is aimed at identifying cellular elements involved in Th-1 polarized immune pathways that are triggered by the recognition of inactivated virus particles. In attempts to dissect the earliest response-shaping events after immunisation we assessed the activating effects of non-replicating virions on plasmacytoid and myeloid DCs. In addition, we studied the capacity of virion-exposed adoptively transferred DCs to generate antiviral IFN- γ -producing cells and IgG2a class switching by B cells. Our data show that early interactions between plasmacytoid/myeloid DCs and non-replicating virions are apparently sufficient to initiate T and B cells responses conditioned towards the Th-1 phenotype. Th-1 polarization is independent of the DC type transferred, and of its capacity to produce IL-12 and IFN- α in vitro. However, the genotype of the recipient may affect the overall efficacy of DC vaccination. Our data are relevant for prophylactic and therapeutic DC-based vaccination regimes.

2. Materials and methods

2.1. Mice

Wild type (WT) mice on a C57BL/6 background were obtained from Jackson Laboratories (Bar Harbor). WT 129/Sv mice were bred at the Central Animal Laboratory, Utrecht University. All mice were housed in filtertop cages. Approval for the animal experiments had been obtained from the Institutional Animal Welfare Committee.

2.2. In vivo treatment of mice

Immunisation studies were performed in 8–12-week old female mice by intraperitoneal (i.p.) injection of 100 μ l iPRV, containing 1×10^7 inactivated plaque-forming units (PFU), or with ex vivo pulsed DCs. In that case 2×10^5 iPRV-pulsed or control DCs were injected i.p. Blood samples of immunised mice were taken at various points in time after vaccination from the retro-orbital plexus.

2.3. Viruses

Because of the high virulence for mice of wild type PRV, an attenuated gE $^{-}$, TK $^{-}$ mutant was used as live PRV control to infect DCs [17]. A gE $^{-}$, TK $^{+}$ derivative of the field isolate NIA-3 [18] was used to prepare iPRV. Inactivation was performed using a final concentration of 0.01 M 2-bromoethylamine hydrobromide (BEA) and NaOH, pH 7.4, for 24 h at 37 °C. BEA was neutralized by adding 20% sodium thiosulphate to a final concentration of 0.67% (v/v). No residual live virus was found, as assessed by inoculation of BHK cell cultures (>1500 cm 2 monolayer surface) with the preparation and checking for cytopathic effects. After dialysis against phosphate buffered saline (PBS), iPRV was used to stimulate DCs and immunize mice.

2.4. Cell isolation and DC culture

Bone marrow cells were isolated by flushing femurs and tibiae from 5–10 naïve mice with cold RPMI. Erythrocyte-depleted cells were passed through a 70 μ m cell strainer and cultured at a concentration of 10^6 cells/ml in RPMI medium [19] supplemented with glutamax-I, 5% FCS, Gentamycine, 5×10^{-5} M β -mercaptoethanol and either 200 ng/ml recombinant human FLT3-Ligand (Peprotech) or 100 ng/ml recombinant murine granulocyte macrophage colony stimulating factor (GM-CSF, Peprotech).

2.5. Flow cytometric analysis and cell sorting

For cell sorting, DCs were harvested at day 8 of culture and stained with FITC labelled mAb anti-CD11c and PE labelled anti-CD11b (both from BD Pharmingen). Plasmacytoid (CD11c $^{+}$ CD11b $^{-}$) cells from the FLT3-L culture and myeloid (CD11c $^{+}$ CD11b $^{+}$) cells from the GM-CSF culture were sorted by FACS [19].

2.6. In vitro stimulation of DCs

Sorted plasmacytoid (CD11c $^{+}$ CD11b $^{-}$) and myeloid (CD11c $^{+}$ CD11b $^{+}$) cells were cultured in the presence of iPRV (1×10^7 inactivated PFU), live attenuated PRV (multiplicity of infection 5, [17]) or in medium as a control. Cytokines produced were quantitated in cell free medium collected after 24 h. Immunisations were performed with 2×10^5 viable PRV-pulsed or control DCs, washed three times with phosphate buffered saline [20].

2.7. Analysis of cytokine production

For the analysis of IL-5 and IFN- γ production, spleens were isolated 2 weeks after immunization. Erythrocyte-depleted splenocytes (5×10^6 cells/ml) were cultured in 96-well plates (Nunc) in the presence of 10 ng/ml dialysed iPRV. Cell culture supernatants were harvested 7 days later, and cytokines were measured using sandwich ELISAs for IL-5

and IFN- γ (both from R&D systems). DC culture supernatants were tested using sandwich ELISAs for IFN- α (PBL-Biomedical) and IL-12. The IL-12p40 ELISA was performed using Greiner Microlon 96 well ELISA plates incubated for 24 h at 4 °C with anti IL-12 clone 15.6 (BD Pharmingen) at a concentration of 4 ng/ml. After blocking with 1% bovine serum albumin, samples and standards (rmIL-12, BD Pharmingen) were incubated overnight at 4 °C, biotinylated anti IL-12 clone 17.8 (BD Pharmingen) was used as detecting antibody at a concentration of 2 ng/ml. After incubation with peroxidase-conjugated streptavidine, tetramethylbenzidine was allowed to react for 10 min. The colour reaction was stopped and the results read at 450 nm.

2.8. Determination of antibody titres

Serum antibody levels and subtypes were determined using ELISA in 96-well flat bottom plates as described before [14]. Antibody titres were defined as the reciprocal of the highest dilution with an absorbance 1.5 times that of the background observed in naive, non-immunised control groups. The detection limit (DL) of the assay was a 2 log 6 titre.

2.9. Statistics

If applicable, group means of cytokine responses and antibody titres were compared using the Student's *t*-test.

3. Results

3.1. Cytokine production by plasmacytoid and myeloid DCs exposed to iPRV

To investigate processes that precede the priming of naive Ag-specific T cells we examined the effects of non-replicating and live virions on in vitro cultured immature bone marrow DCs of either plasmacytoid or myeloid phenotype. Bone marrow cells of naive 129/Sv mice were cultured for 8 days, in the presence of either FLT3-L or GM-CSF, and DC subtypes were sorted using FACS analysis [19]. After 24 h exposure to iPRV or live PRV, culture media of in vitro incubated DCs were examined for cytokine production. As shown in Fig. 1, plasmacytoid DCs (CD11c⁺CD11b⁻), but not myeloid DCs (CD11c⁺CD11b⁺), responded with high IL-12p40 production following stimulation with iPRV, and to a lesser extent with live PRV ($p = 0.0024$, Fig. 4A and B). We analysed the expression of the IL-12p40 subunit as a marker for IL-12p70 production. In cell culture supernatants of unstimulated DCs relatively low amounts of IL-12p40 were found. Similarly, IFN- α was produced by plasmacytoid DCs, but not by myeloid DCs (Fig. 1C and D), which is in line with their plasmacytoid phenotype [19]. Both live attenuated and iPRV induce comparable amounts of this cytokine; indicating that replication of the virion is not required for this DC response. Exposure of both DC subtypes to tetanus toxoid (TT), a prototypic non-viral antigen, evoked no IL-12 nor IFN- α/β production (data not shown). IFN- γ could not be detected in any of the cell supernatants (data not shown).

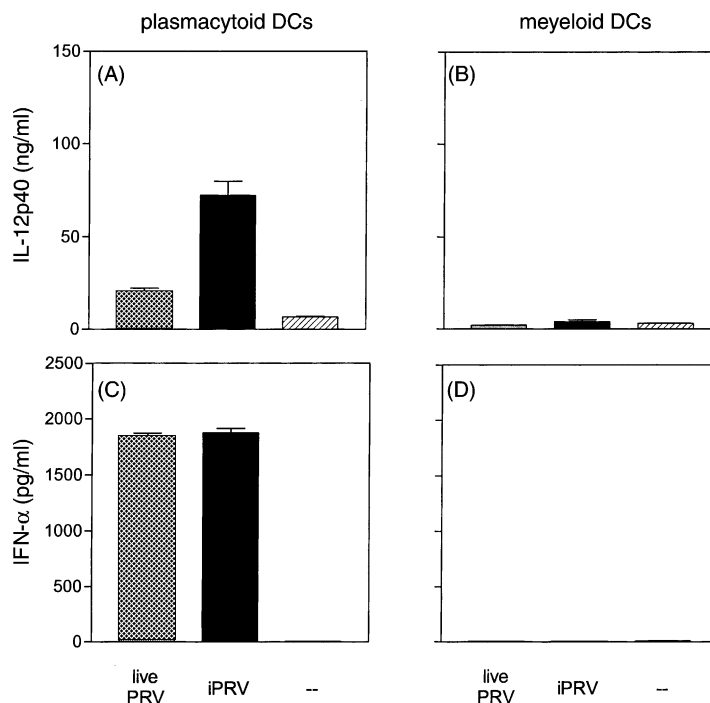


Fig. 1. In vitro cytokine responses of different types of DCs isolated from 129/Sv mice following stimulation with live or iPRV. IL-12 (A, B) and IFN- α (C, D) production was measured in cell free supernatants of either plasmacytoid (A, C) or myeloid (B, D) DCs using ELISA. Mean concentrations of at least three individual cultures per group are shown \pm S.E.M.

3.2. 129/Sv mice vaccinated with PRV-loaded DCs develop type 1 adaptive immune responses to PRV

In order to reveal their *in vivo* immune inductive and putative response skewing capacity following virion exposure, plasmacytoid or myeloid DCs, *in vitro* stimulated with PRV for 24 h were washed and injected into naive mice. The medium in which the DCs were resuspended to inject mice contained less than 10 PFU PRV as determined by plaque titration, a dose that by itself did not induce detectable primary immune responses (not shown). Two weeks later Ag-specific splenic cytokine responses and Ag-specific serum antibody response were measured in DC vaccinates. As a control, mice were immunised with iPRV directly.

Despite differences in cytokine profiles between 129/Sv DC subsets following *in vitro* exposure to live or iPRV, recipients of *in vitro*-pulsed DCs of both subtypes generated similar immune reactions. Ag-specific cytokine responses of *ex vivo* restimulated splenocyte cultures, characterised by high IFN- γ and low IL-5 levels, were noted for recipients of both DC subtypes, regardless of exposure to live or iPRV (Fig. 2). On the other hand, vaccination with TT-loaded DCs induced no detectable T cell derived cytokine response (data not shown).

The antibody quality resulting from immunisation with both DC subsets appeared to be in line with the splenic cytokine profile. Except for mice immunised with non-stimulated control DCs, all animals responded with markedly high IgG2a antibody levels and low IgG1 titres (Fig. 3). These data illustrate that the type of DC used for vaccination, does not influence the antiviral antibody isotype distribution in DC recipients. After vaccination with TT-loaded DCs no TT specific primary antibody responses were detected (not shown).

3.3. Vaccination with PRV-loaded DCs, of either plasmacytoid or myeloid origin, does not trigger significant antibody and T cell responses in C57BL6 mice

We noted earlier that the observed Th-1 pathway may be activated differently in genetically different hosts [43]. We

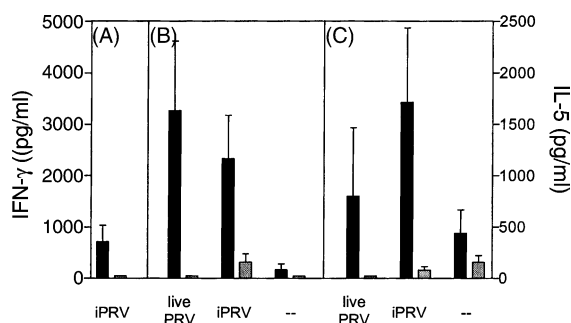


Fig. 2. Splenic cytokine responses of 129/Sv vaccines 2 weeks after immunization with (A) iPRV directly, or (B) with *in vitro* PRV-loaded plasmacytoid or (C) myeloid DCs. Mean IFN- γ (left axis, solid columns) and IL-5 (right axis, grey columns) concentrations of five mice per group are shown \pm S.E.M.

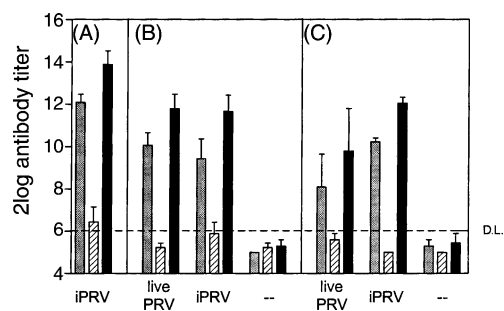


Fig. 3. PRV specific antibody responses in 129/Sv mice following vaccination with *in vitro* PRV-pulsed DCs. Sera of five mice per group taken 2 weeks after immunization with (A) iPRV directly, (B) PRV loaded plasmacytoid or (C) myeloid DCs, were analysed for specific IgG (H+L) (grey columns), IgG1 (hatched columns) and IgG2a (solid columns). Mean titres \pm S.E.M. are shown, the detection limit (DL) is indicated by the dotted line.

therefore performed similar experiment in C57BL/6 mice. When using C57BL/6 mice as bone marrow donor, both plasmacytoid DCs cultured in the presence of FLT3-L and remarkably also myeloid DCs cultured in the presence of GM-CSF responded with IL-12 production to iPRV (Fig. 4A and B). IL-12 production upon stimulation with inactivated particles was higher than when plasmacytoid DCs were stimulated with live PRV ($p = 0.015$, Fig. 4A). Conversely, IFN- α production was noted only for plasmacytoid DCs exposed to either replicating or iPRV (Fig. 4C), but minimal for myeloid DCs (Fig. 4D). IFN- γ could not be detected in any of the cell supernatants (not shown). When vaccinated with iPRV, C57BL/6 mice responded with significant IgG and IgG2a responses (not shown). However, immunization with *in vitro* iPRV or live PRV-pulsed DCs of either subtype did not induce significant T cell nor antibody responses (Figs. 5A, B and 6A, B).

3.4. Vaccination of 129/Sv mice with PRV-loaded DCs of C57BL6 origin triggers Th-1 type adaptive T and B cell responses to PRV

In view of the strain differences in response to autologous DC immunization between 129/Sv and C57BL/6 mice we decided to evaluate the responses of well-responding 129/Sv mice to immunization with DCs of C57BL/6 origin. Because the MHC class II of C57BL/6 and 129/Sv mice are genotypically identical [21], *in vitro* pulsed DCs of C57BL/6 origin were used to immunize 129/Sv mice. Remarkably, 129/Sv mice clearly responded with type 1 immune responses (Figs. 5A, B, 6A and B), as they did when receiving iPRV-pulsed autologous DCs (Figs. 2 and 3). In both situations mainly IFN- γ (Fig. 5) and IgG2a type antibodies (Fig. 6) are produced. The limited T and B cell responses noted following autologous DC vaccination in C57BL/6 mice suggests that the efficacy of DC vaccination to a great extent depends on genetic factors of the vaccinee.

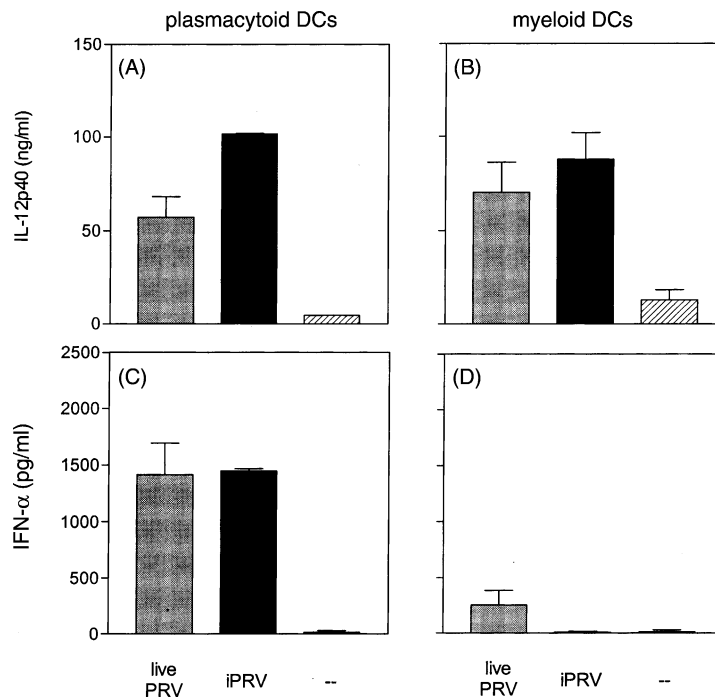


Fig. 4. In vitro cytokine production by plasmacytoid (A, C) or myeloid (B, D) DCs, isolated from C57BL/6 mice, following stimulation with PRV. Cell free supernatants of either DC subtype were tested for IL-12 (A, B) and IFN- α (C, D) using ELISA. Mean cytokine concentrations of at least five mice per group are shown \pm S.E.M.

4. Discussion

Activated T cells are normally generated from resting T cells that require stimulation by antigen-presenting DCs. In this report we show that adoptive transfer of DCs pulsed with inactivated virus particles is sufficient to shape IFN- γ -associated antiviral IgG2a synthesizing B cell responses in naive vaccinates. Interestingly, the use of distinctly generated DC subtypes, exhibiting different IL-12 and IFN- α/β responses in vitro, did not influence the outcome of Th-1 cell polarization and associated antibody isotype patterns in immunised recipients.

Viruses and virus-like particles are known to trigger activation and antigen-presentation by DCs [6,22–24]. We assume that early after inoculation α herpesvirions are internalised and processed by DCs resulting in subsequent presentation to naive helper cells. Likely, only a limited, undefined, fraction of viral antigens is taken up by the in vitro-exposed DCs and apparently sufficient to evoke primary adaptive responses, in contrast to the TT antigen. In general, little is known about the most up-stream molecular interactions leading to DC activation by viral structures. Although several pattern recognition receptors (PRR) for virus-specific structures have been described, their exact role in the induction of innate and subsequent acquired antiviral immune responses still has to be revealed [25,26]. Virus-related pathogen-associated molecular structures that have been defined, such as for the fusion protein of respiratory syncytial virus and for the hepatitis C virus core protein, are both involved in Toll-like receptor (TLR) 4 dependent responses [27–29]. Also more

general motifs associated with virus particles and infection are thought to be recognized; TLR9 and TLR3 bind double stranded DNA and RNA, respectively [30]. Besides, members of the C-type lectin family can ligate virus-specific carbohydrate patterns, leading to maturation and antigen presentation by the DC [31]. However, how exactly iPRV activates DCs to provide presumed costimulatory signals to helper T cells is unknown. Remarkably, the particulate form of the virions and their highly organized structure seemed to contribute to in vitro DC activation [32]. We observed 26–63% lower cytokine levels produced upon stimulation with sodium deoxycholate (NaDOC) disrupted virus particles (data not shown). The influence of the antigenic structure on in vitro immune stimulation has been described before for influenza virus. Whole inactivated influenza virus led to functional DC maturation, which proved moderate following exposure to subunit antigens lacking the core protein [33].

Primary B cell activation requires recognition of native antigen. One may question how vaccination with extensively washed DCs, pulsed overnight with iPRV, enables B cell IgG responses in naive recipients. Wykes and co-workers showed that in vitro addition of free antigen to B cells cultures results in secretion of only IgM, when help from antigen-specific T cells is provided, while B cells precultured in direct contact with Ag-pulsed DCs readily produce both IgM and IgG [34]. It is well recognised that DCs are able to retain model antigens such as horse radish peroxidase (HRP), KLH and human serum albumin in their native form for at least 24 h [34]. For immunisation with Ag-pulsed DCs this appears long enough to migrate to lymph nodes and to provide antigen plus

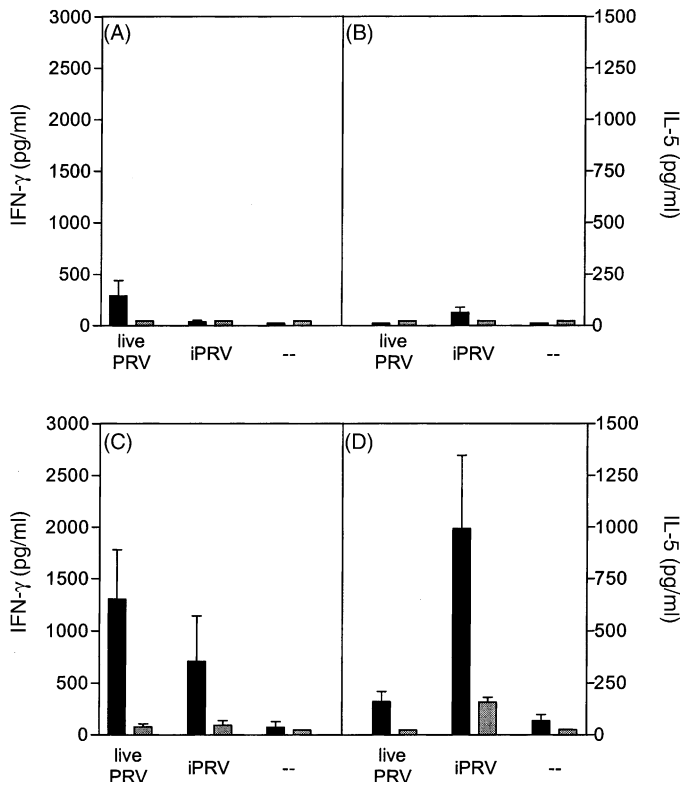


Fig. 5. Splenic cytokine responses of C57BL/6 vaccinates, 2 weeks after immunization with in vitro (A) PRV-pulsed plasmacytoid or (B) myeloid DCs and splenic cytokine responses of 129/Sv mice following adoptive transfer of (C) plasmacytoid or (D) myeloid in vitro PRV-pulsed DCs of C57BL/6 origin. Mean IFN- γ (left axis, solid columns) and IL-5 (right axis, grey columns) concentrations of at least five mice per group are shown \pm S.E.M.

contact-dependent signals essential for IgG class switching. This process, however, remains dependent on additional T cell help [34]. In contrast, IgM induction by iPRV is T cell independent (De Wit, unpublished observations). How exactly DCs expose B cells to antigens remains elusive; we may exclude a carry over effect after extensive washings. Hence, DCs not only initiate T helper activity but also directly initiate and influence antibody synthesis by B cells [34–36]. We assume that similar processes take place in the iPRV system.

Moser and colleagues have shown that the type of Ag-pulsed APC used for adoptive transfer immunisation may affect the subclass of antibody produced. [35]; DCs pulsed with human γ globuline (HHG) stimulated Th-1 priming, while HHG-pulsed macrophages appeared to prime Th-2 development and production of IgG1 and IgE antibodies, but only after a booster was given to the mice after the DC transfer [5,37]. One paradigm advocates that lymphoid DCs initiate Th-1 differentiation, while myeloid DCs preferentially stimulate Th-2 development [4,5,38]. Especially, plasmacytoid murine DCs produce large amounts of IFN- α in response to irradiated HSV, while myeloid DCs fail to produce IFN- α following recognition of irradiated HSV [19]. This is in line with our iPRV data, for DCs derived from both 129/Sv mice and C57BL/6. Non-plasmacytoid DCs can produce IFN- α

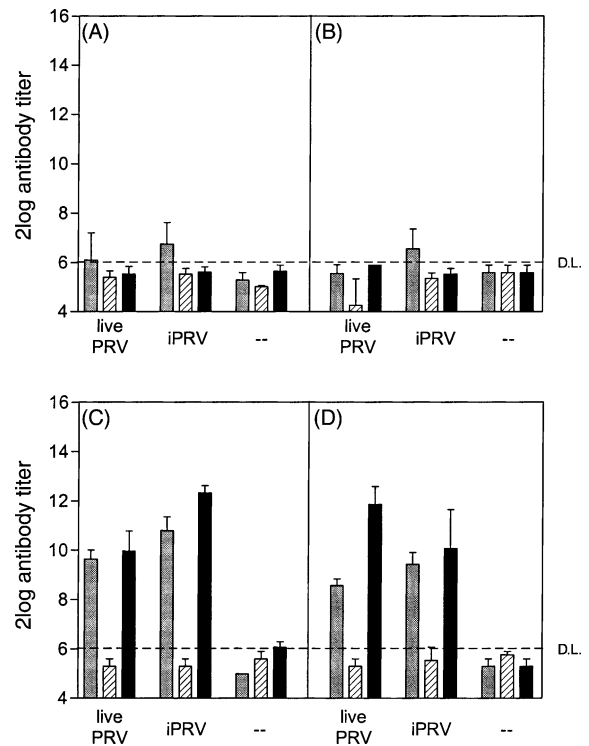


Fig. 6. PRV specific antibody reactions of C57BL/6 mice following immunization with (A) plasmacytoid or (B) myeloid in vitro PRV-loaded DCs and PRV specific antibody responses in 129/Sv mice after immunization with in vitro PRV-loaded DCs of C57BL/6 origin. (C) Plasmacytoid DCs, (D) myeloid DCs. Sera of five mice per group taken 2 weeks after immunization were analyzed for specific IgG (H+L) (grey columns), IgG1 (hatched columns) and IgG2a (solid columns). Mean titers \pm S.E.M. are shown, the detection limit (DL) is represented by a dotted line.

if dsRNA is able to access the cytosol [6]. Myeloid DCs from 129/Sv and C57/BL6 mice showed different IL-12 producing capability. iPRV induces IL-12 in both, plasmacytoid and remarkably myeloid DCs of C57BL/6 origin, while only plasmacytoid DCs from 129/SV mice produced IL-12. The fact that the iPRV preparation contained more virions may explain some of the quantitative differences observed in the production of IL-12. Since the function of the two DC subtypes is thought to be reflected in the expression of different sets of PRR [30,39], we speculate that either both DC subtypes recognize distinct viral structures, or that both DC types recognize common viral patterns, whose receptors have yet to be revealed. Importantly, our data show that regardless of the recognition process both plasmacytoid and myeloid DC subtypes are able to evoke similar serum IgG isotype patterns, consistent with expansion of IFN- γ producing Th-1 cells. Thus, we conclude that the antigen rather than the nature of DCs dictates the type of antiviral immune responses. Moreover, DC activation in our system is independent of viral replication, implicating that recognition of the virus particle itself is sufficient to initiate and skew downstream adaptive responses.

Although it has been shown that Th-1 predominance, consistent with IgG2a isotype synthesis, depends on IL-

12 [4,5,40] this cytokine was not produced in vitro by myeloid 129/Sv DCs upon PRV stimulation. However, since these DCs, similar to the high IL-12 producing lymphoid DCs, equally induced Th-1 differentiation following adoptive transfer, we conclude that IL-12 is not a major factor for Th-1 development evoked by iPRV. Also the virus-induced levels of in vitro IFN- α production by DCs, high in plasmacytoid cultures but minimal or absent in myeloid cultures, could not be linked to in vivo Th-1 development and associated IgG class switching. These findings are consistent with our earlier reports, using relevant gene-targeted mice, showing that IL-12, IL-23, IFN- γ and IFN- α/β independent type 1 immune responses occur following live virus infections or direct immunisation with inactivated viruses in absence of exogenous vaccine adjuvant [13,14,16]. Interestingly, IL-12 independent type-1 immunity was observed for other viral infections [8,9,11,12]. These data observed in mice largely fit with observations in patients carrying mutations in the IL-12 p40 or the IL-12 receptorp1 gene, who respond normally to viral vaccines, but have difficulties to control *Salmonella* and mycobacterial infections [41].

Interestingly, vaccination with iPRV pulsed C57BL/6 DCs did not induce measurable immune responses when injected into C57BL/6 mice, but readily evoked Th-1 associated antibody and cytokine reactions when transferred into 129/Sv mice. This indicates that not only DC activation but also the host responsiveness critically mediates down-stream immune responses. Expression of inflammatory cytokines like CCR7 may be critical for DC based vaccination [42].

In conclusion, inoculation of non-replicating α -herpes virions conditions both plasmacytoid and myeloid DCs to initiate Th-1 type T cells responses and associated IgG class switching by B cells. The exact molecular basis of this type 1 response skewing viral stimulus remains subject for future studies.

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