elicited by 10 μM 5-HT applied to outside-out membrane patches. Data analysis was performed with the Electrophysiology Data Recorder developed and provided by J. Dempster (Department of Physiology and Pharmacology, University of Strathclyde, UK; http://www.strath.ac.uk/Departments/PhysPharm/). Data are reported as means ± s.e.m.

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Viral infection switches non-plasmacytoid dendritic cells into high interferon producers

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Type I interferons (IFN-I) are important cytokines linking innate and adaptive immunity[1]. Plasmacytoid dendritic cells make high levels of IFN-I in response to viral infection and are thought to be the major source of the cytokines *in vivo*[2]. Here, we show that conventional non-plasmacytoid dendritic cells taken from mice infected with a dendritic-cell-tropic strain of lymphocytic choriomeningitis virus make similarly high levels of IFN-I on subsequent culture. Similarly, non-plasmacytoid dendritic cells secrete high levels of IFN-I in response to double-stranded RNA (dsRNA), a major viral signature[3], when the latter is introduced into the cytoplasm to mimic direct viral infection. This response is partially dependent on the cytosolic dsRNA-binding enzyme protein kinase R[4] and does not require signalling through toll-like receptor (TLR) 3, a surface receptor for dsRNA[5]. Furthermore, we show that sequestration of dsRNA by viral NS1 (refs 6,7) explains the inability of conventional dendritic cells to produce IFN-I on infection with influenza. Our results suggest that multiple dendritic cell types, not just plasmacytoid cells, can act as specialized interferon-producing cells in certain viral infections, and reveal the existence of a TLR-independent pathway for dendritic cell activation that can be the target of viral interference.

Although all cells can produce IFN-I, plasmacytoid dendritic cells (PDCs) produce 1,000-fold higher levels than other cell types, and are responsible for systemic IFN-I responses to many viruses[2]. However, depletion experiments show that PDCs are not essential for the IFN-I response to the Armstrong strain of lymphocytic choriomeningitis virus (LCMV)[8], suggesting the existence of alternative interferon-producing cells *in vivo*. These could include conventional, non-plasmacytoid dendritic cells found at all potential sites of pathogen entry. To address this possibility, we purified conventional non-plasmacytoid $CD11c^{high}$ $Ly6C^-$ $B220^-$ dendritic cells from the spleen of mice infected with LCMV intravenously, and measured IFN-α secretion after culture *in vitro*. We used two LCMV isolates, the Armstrong strain and an Armstrong variant, clone 13, which exhibit a different tropism: three days after intravenous inoculation, clone 13 can be found within dendritic cells in T-cell areas of the spleen, whereas Armstrong replicates predominantly in other cell types in the red pulp[9]. As reported previously[8], IFN-α production was not detected on culture of conventional $CD11c^{high}$ dendritic cells from mice infected with LCMV Armstrong (Fig. 1a). However, although little type 1 IFN messenger RNA was found in dendritic cells from clone-13-infected mice immediately on isolation (data not shown), high levels of IFN-α were produced when

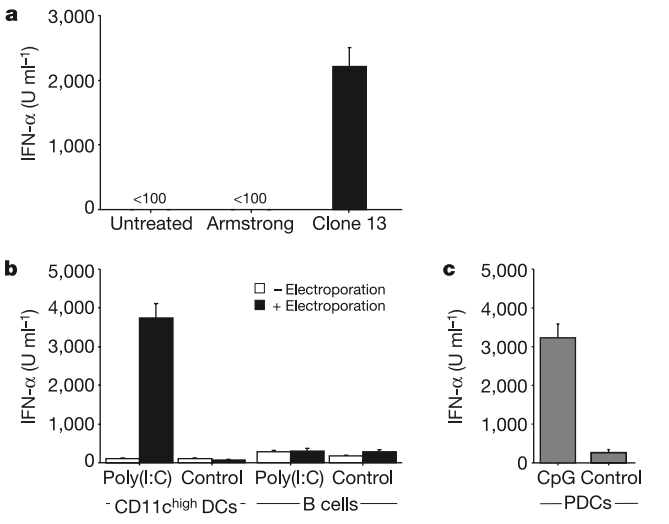

Figure 1 Viral infection or cytosolic dsRNA can induce high levels of IFN-α production by non-plasmacytoid splenic dendritic cells. **a**, C57BL/6 mice were infected intravenously with LCMV Armstrong or clone 13. Splenic CD11c^{high} dendritic cells were purified three days after infection, cultured overnight, and IFN-α accumulation in the supernatant was measured. Data are representative of three independent experiments. **b**, Splenic CD11c^{high} dendritic cells (DCs) (BALB/c) or B cells (C57BL/6) were electroporated in the presence or absence of 3 μg poly(I:C) or were exposed to the electroporation buffer with or without poly(I:C), but not subjected to the electrical pulse, before being cultured overnight in medium alone. Data show IFN-α accumulation in supernatants. **c**, Sorted CD11c^{dim} Ly6C^{+} B220^{+} spleen PDCs (BALB/c) were cultured overnight with or without CpG (0.5 μg ml^{-1}), and secreted IFN-α was measured as above. Data represent the average values of triplicate samples ±1 s.d. and are representative of at least four independent experiments.

these dendritic cells were subsequently cultured *in vitro* (Fig. 1a). These results demonstrate that conventional non-plasmacytoid dendritic cells have the potential to produce high levels of IFN-I after viral exposure and, therefore, suggest that the ability to act as interferon-producing cells in viral infections may not be restricted to a single dendritic cell subtype.

The viral components leading to IFN-I induction remain largely unidentified[10]. Among the best known is dsRNA, an intermediate in the replicative cycle of many viruses[3]. To dissect some of the events linking viral recognition to IFN-I production, we examined the response of non-plasmacytoid dendritic cells to the synthetic dsRNA polyriboinosinic polyribocytidylic acid (poly(I:C)). CD11c^{high} Ly6C^{-} B220^{-} dendritic cells treated with poly(I:C) made only background levels of IFN-α (Fig. 1b). However, because viral replication takes place in the cytosol, we then assessed the effect of delivering poly(I:C) intracellularly. Electroporation with poly(I:C) allowed conventional splenic dendritic cells to produce levels of IFN-α much greater than previously reported[11] and in the same order of magnitude as those made by PDCs (Fig. 1b, c). In contrast, B cells (Fig. 1b) or fibroblasts (not shown) did not produce high levels of IFN-α under the same conditions. To exclude the possibility of a PDC contaminant in the CD11c^{high} spleen fraction, experiments were repeated with CD11c^{high} dendritic cells grown *in vitro* from bone marrow precursors (BM-DCs) using granulocyte macrophage colony-stimulating factor (GM-CSF). BM-DCs also made high amounts of IFN-α after electroporation with poly(I:C) or after treatment with poly(I:C) in combination with lipofectamine, a reagent for nucleic acid transfection (Fig. 2a, b). The same treatments also induced IFN-β mRNA expression (data not shown). Lipofectamine did not simply act as a nonspecific potentiator of cytokine synthesis, as interleukin-12 (IL-12) p70 induction actually decreased in the presence of the lipid (Fig. 2c). Similar to poly(I:C), dsRNA isolated directly from a reovirus also induced high levels of IFN-α production by BM-DCs when administered by electroporation or together with lipofectamine but not

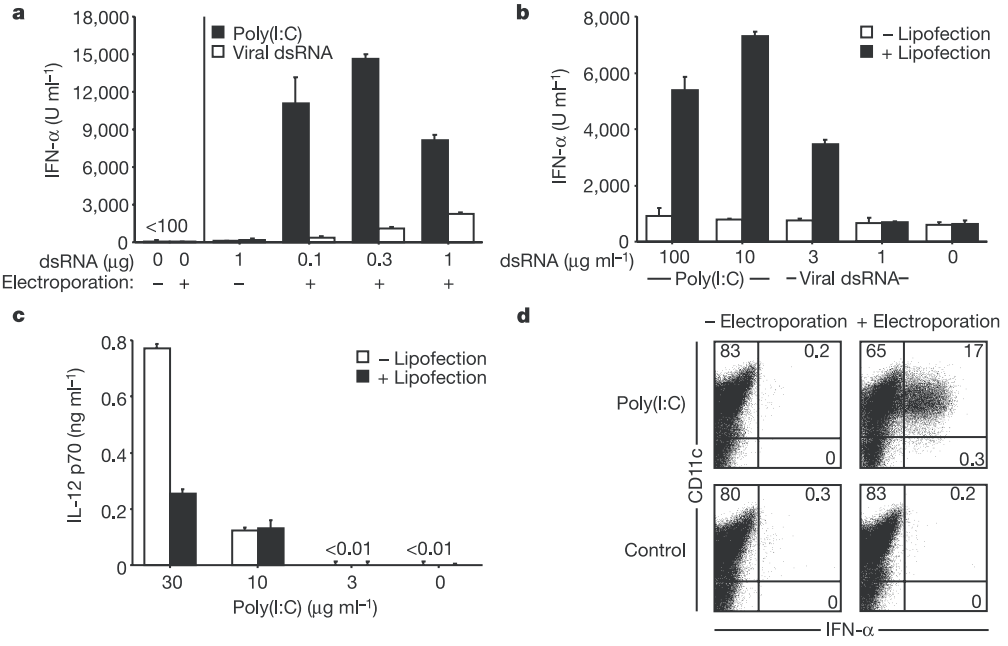

Figure 2 Cytosolic administration of dsRNA induces IFN-α production by BM-DCs. **a**, C57BL/6 BM-DCs were electroporated or not with the indicated amounts of poly(I:C) or reoviral dsRNA as in Fig. 1b. **b**, C57BL/6 BM-DCs were treated with or without different concentrations of poly(I:C) or viral dsRNA in the presence or absence of lipofectamine. **a**, **b**, IFN-α in culture supernatants was measured by ELISA after overnight culture. **c**, As for **b** but measuring IL-12 p70. Data in **a**–**c** represent the average values of triplicate samples ±1 s.d. and are representative of at least three independent experiments. **d**, Intracellular IFN-α staining of C57BL/6 BM-DCs after electroporation with 0.3 μg poly(I:C). Numbers indicate percentage of total cells in each quadrant. Data are representative of three independent experiments.

when given exogenously (Fig. 2a, b; the lower levels of IFN-α induced by reoviral dsRNA are probably due to the fact that it could not be used at saturation because of limited availability). Notably, IFN-α production in BM-DC cultures was not attributable to a small number of contaminating PDCs, as 14–22% of $CD11c^{high}$ cells stained for the cytokine after electroporation with poly(I:C) (Fig. 2d). IFN-α production by BM-DCs in response to cytosolic dsRNA could be amplified but not initiated by providing a co-signal via CD40 (Supplementary Fig. 1), suggesting that it might be further regulated during dendritic cell–T-cell interactions, as reported for other cytokines[12]. We conclude that non-plasmacytoid dendritic cells can act as interferon-producing cells in response to dsRNA if the viral pattern crosses the plasma membrane.

TLR3 can mediate innate stimulation by dsRNA, and it is important for the induction of IFN-α and IFN-β genes in macrophages and for IL-12 p40 production by BM-DCs in response to poly(I:C)[5]. Notably, TLR3 message was undetectable in resting immature BM-DCs (Supplementary Fig. 2a, b). Nevertheless, activation of BM-DCs with a variety of stimuli, including poly(I:C), caused an increase in TLR3 mRNA expression (Supplementary Fig. 2b). To determine whether this might account for the IFN-I response, $TLR3^{-/-}$ BM-DCs were treated with poly(I:C) plus lipofectamine and assayed for IFN-α secretion. As shown in Fig. 3a, TLR3 deficiency did not impair IFN-α production. To examine the involvement of other TLRs, we tested the requirement for MyD88, a TLR adapter[13]. $MyD88^{-/-}$ BM-DCs mounted normal IFN-α responses to poly(I:C) plus lipofectamine (Fig. 3b). IFN-α induction was also resistant to the effects of a peptide originally believed to interfere specifically with TIRAP function but now known to block other pathways, including MyD88-independent TLR4 signalling, which can lead to IFN-I gene expression[14] (Fig. 3b). These results, together with the known topology of TLRs (ligand-binding domain facing away from the cytosol), suggest that IFN-α produced by dendritic cells in response to cytosolic dsRNA is TLR-independent.

The apparent lack of TLR involvement and requirement for cytosolic delivery suggested that IFN-α production might require recognition of dsRNA by an intracellular pattern recognition receptor. Protein kinase R (PKR) is a cytosolic serine/threonine kinase activated by autophosphorylation on binding to dsRNA[4]. Although PKR is primarily involved in restricting viral replication[4], it has also been implicated in IFN responses to some viruses[15] and in IFN-I induction by dsRNA in cell lines[16–19]. Consistent with this role, 2-aminopurine, a PKR inhibitor[16,17], blocked IFN-α production by BM-DCs after treatment with poly(I:C) plus lipofectamine, even when CD40L was used to provide maximum amplification (Supplementary Fig. 3). The effect was selective as 2-aminopurine did not inhibit production of IL-12 p70 in response to CpG-containing oligonucleotide (CpG) or lipopolysaccharide (LPS) (see Supplementary Fig. 3). To formally implicate PKR, the experiments were repeated using BM-DCs grown from mice lacking the PKR dsRNA-binding domain ($PKR^{-/-}$ mice[18]). $PKR^{-/-}$ BM-DCs displayed a marked (although not absolute) reduction in IFN-α production after stimulation with poly(I:C) plus lipofectamine and CD40L (Fig. 3c). In contrast, IL-12 p70 production was actually increased in $PKR^{-/-}$ dendritic cells (Fig. 3c), possibly because negative regulation by IFN-I was abrogated[20]. We conclude that PKR has an important function in the IFN-I response of dendritic cells to cytosolic dsRNA but is not required for production of IL-12.

Many viruses have evolved strategies to avoid activation of PKR[10]. For example, influenza, widely used to stimulate production of IFN-I by PDCs[21–25], encodes the NS1 protein, which sequesters dsRNA[7]. To test the hypothesis that NS1 blocks the IFN-I response of non-plasmacytoid dendritic cells, we compared IFN-α production by BM-DCs in response to infection with wild-type

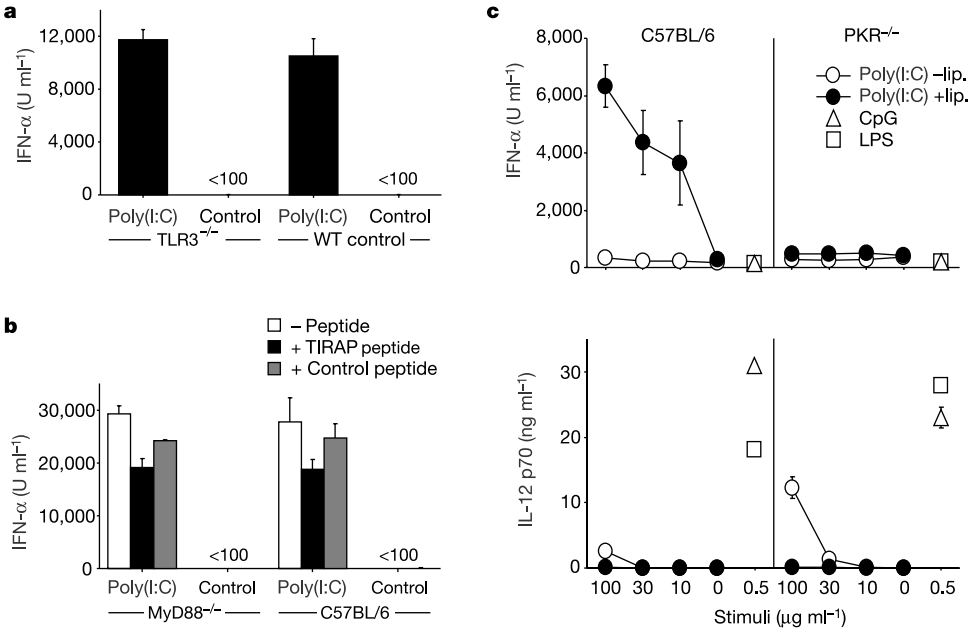

Figure 3 IFN-α induction by dsRNA does not require TLR signalling but is largely dependent on PKR. **a**, BM-DCs from $TLR3^{-/-}$ or control mice were treated with poly(I:C) (30 μg ml^{-1}) plus lipofectamine or left untreated (control). Cells were cultured on CD40L-expressing fibroblasts and IFN-α accumulation in culture supernatants was determined after overnight culture. **b**, $MyD88^{-/-}$ or control wild-type (WT) BM-DCs were treated as in **a** in the presence of an inhibitory peptide (TIRAP), control peptide or in the absence of peptide. Data represent the average values of triplicate samples ±1 s.d. and are representative of three independent experiments. The inhibitory peptide but not the control peptide prevented upregulation of CD86 in dendritic cells treated with LPS (data not shown; see also ref. 12). **c**, BM-DCs from $PKR^{-/-}$ or wild-type littermate control mice were treated with or without the indicated concentrations of poly(I:C) in the presence or absence of lipofectamine, or with CpG or LPS (both at 0.5 μg ml^{-1}) in the absence of lipofectamine. Fc–CD40L was used throughout to amplify cytokine production. IFN-α and IL-12 p70 accumulation in supernatants was determined after overnight culture. Data represent the average values of triplicate samples ±1 s.d. and are representative of four independent experiments.

influenza or with an NS1-deleted mutant virus (ΔNS1)[6]. Consistent with previous reports[21–25], non-plasmacytoid BM-DCs produced little IFN-α in response to wild-type influenza despite the fact that the virus infected up to 60% of the cells as assessed by staining for influenza nucleoprotein (NP) (Fig. 4a, b). However, infected BM-DCs also expressed high levels of NS1 (Fig. 4a). In contrast, infection with ΔNS1 influenza induced production of high levels of IFN-α (Fig. 4b). This was especially striking as ΔNS1 replicates poorly in wild-type cells[6,7] and infected only a small fraction of BM-DCs (<10% NP^+ cells) (Fig. 4a). We conclude that dsRNA produced during active viral replication in conventional dendritic cells can trigger high levels of IFN-α production provided that the virus does not actively interfere with dsRNA recognition pathways.

Our data suggest that non-plasmacytoid dendritic cells can function as interferon-producing cells after viral infection, and demonstrate that there are at least two pathways for their activation by viral dsRNA. Cell surface recognition through a PKR-independent pathway is sufficient to trigger production of IL-12 (Fig. 3c). In contrast, IFN-I production requires access of dsRNA to the cytoplasm and involves PKR and, possibly, related kinases. These results imply that conventional dendritic cells can discriminate between direct infection with actively replicating virus compared with contact with free viral particles or virally infected cells. Notably, previous investigations have failed to reveal non-plasmacytoid dendritic cells as major IFN-I producers in response to viral infection[2]. In some instances, this may have been due to lack of viral tropism for dendritic cells, as suggested by our experiments comparing LCMV Armstrong and clone 13 (Fig. 1a). In other cases, it is likely to have been due to lack of viral replication and consequent lack of dsRNA production (for example, experiments using inactivated viruses[26,27]) or to viral sequestration of the stimulus as shown by our experiments with ΔNS1 influenza (Fig. 4). In contrast to conventional dendritic cells, PDCs respond both to wild-type influenza and inactivated virus[21–25,27] and must, therefore, possess additional, dsRNA-independent pathways for IFN-I induction. Thus, the fundamental difference between plasmacytoid and non-plasmacytoid dendritic cells seems to be in the mechanisms used for coupling viral recognition to IFN-I synthesis rather than in an ontogenetically determined ability to produce vast amounts of the cytokines.

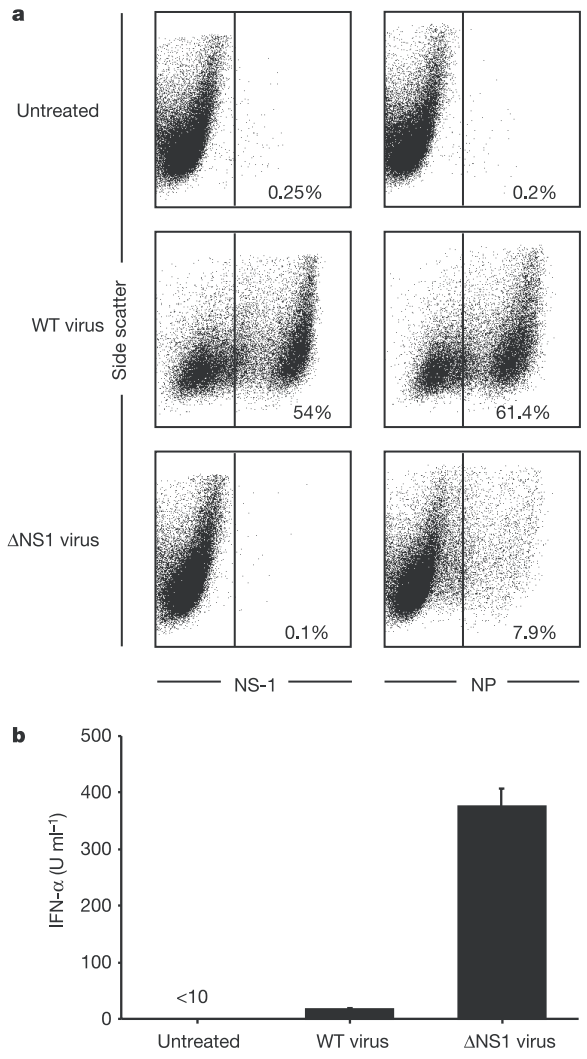

Figure 4 Infection with ΔNS1 influenza allows IFN-α production by non-plasmacytoid dendritic cells. **a**, BM-DCs were infected with influenza ΔNS1 or wild-type virus or were left untreated. Cells were stained for influenza NS1 and NP protein after overnight culture and analysed by flow cytometry after gating on $CD11c^{high}$ cells. **b**, Same cells as in **a**. IFN-α accumulation in the supernatant was measured after overnight culture. Data are representative of three independent experiments.

PKR has been implicated previously in IFN-I production by fibroblasts in response to exogenously added dsRNA[16–19]. However, because TLR signalling can lead to PKR activation[14], this could have been due to TLR3 engagement at the cell surface[10]. Here, we demonstrate that PKR can participate in induction of high levels of IFN-I independently of TLR3 or other TLRs. This may involve PKR itself acting as a cytosolic pattern recognition receptor for dsRNA or might require an upstream dsRNA-binding PKR activator such as PACT[4]. PKR can then lead to the activation of a pathway leading to phosphorylation of IRF3 and IRF7 (refs 4,19), two transcription factors critical for the expression of IFN-β/IFN-α_4 and non-IFN-α_4 genes, respectively[28]. In contrast to fibroblasts[28], BM-DCs, splenic $CD11c^{high}$ dendritic cells and splenic PDCs constitutively express mRNA for IRF3 and IRF7 (data not shown). Therefore, it is likely that most dendritic cell types can rapidly make all IFN-α subtypes on stimulation and not just the IRF3-dependent IFN-β/IFN-α_4.

Other pathways besides PKR probably have a role in IFN-I induction in infected dendritic cells. Indeed, $PKR^{-/-}$ dendritic cells still produce IFN-α after transfection with high levels of dsRNA (data not shown), suggesting that PKR deficiency decreases sensitivity to the stimulus (Fig. 3) but does not abrogate the response entirely. Unfortunately, this has prevented us from assessing the degree to which the IFN-I response to ΔNS1 influenza is PKR-dependent. Comparing wild-type and $PKR^{-/-}$ dendritic cells is uninterpretable because ΔNS1 virus replicates to 10^5 higher titres in PKR-deficient cells[7]. This generates high levels of dsRNA, which can overwhelmingly induce IFN-α production through PKR-independent pathways even if these pathways normally account for only a fraction of the IFN-I response. Such induction of IFN-I is not seen during infection with wild-type influenza presumably because dsRNA sequestration by NS1 prevents activation of all cytosolic dsRNA recognition pathways. Similar considerations may explain the data reported by ref. 19, that $PKR^{-/-}$ fibroblasts show impaired IRF3/7 phosphorylation and IFN-I induction in response to dsRNA but not to infection with Newcastle disease virus (NDV). Although this discrepancy was originally attributed to IFN-I induction by NDV components other than dsRNA[19], it may equally have been the result of unchecked viral replication in PKR-deficient cells generating levels of dsRNA sufficient to activate secondary pathways for IFN-I induction. Consistent with this interpretation, NDV-induced IRF3/7 phosphorylation was completely blocked by E3L, a vaccinia dsRNA-sequestering protein similar to NS1 (ref. 19). These arguments justify our preference for delivering controlled amounts of dsRNA rather than using virus infection to dissect IFN-I production pathways in dendritic cells. Independent of its role during viral infection, the demonstration of a TLR-independent mechanism for

cytosolic dsRNA recognition via PKR, leading to production of high amounts of IFN-I by conventional dendritic cells, suggests that cytosolic targeting of nucleic acids could be useful in vaccination and/or immunotherapy. □

Methods

Reagents

Poly(I:C) was from Pharmacia. LPS (*Escherichia coli* serotype 0128:B12) and 2-aminopurine were from Sigma. Recombinant murine GM-CSF, CpG-containing oligonucleotide and the TIRAP inhibitory and control peptides[14] were made at Cancer Research UK. Reoviral dsRNA was purified from cells infected with bluetongue virus serotype 10. Soluble trimeric CD40L contained the rat CD4 leader, the α-helical coiled-coil domain of human lung surfactant protein D (amino acids 223–257) and the extracellular domain of murine CD154 (amino acids 50–260). Fc–CD40L contained the extracellular domain of murine CD154 and a modified human immunoglobulin-γ1 (IgG1) crystallizable fragment (Fc) region. All reagents were free of endotoxin.

Cells

C57BL/6 mice were obtained from Charles River or from the Institute for Animal Health. BALB/c and MyD88$^{-/-}$ mice[13] (a gift from S. Akira) were bred at Cancer Research UK. PKR$^{-/-}$ mice[18] were bred at the University of Veterinary Medicine, Vienna, Austria. TLR3$^{-/-}$ mice[5] were bred at Yale University. Splenocytes were prepared by digestion with Liberase CI and DNaseI[12]. PDC and conventional CD11c^{high} dendritic cells were purified from splenocyte suspensions as described[29]. Sorted dendritic cells were 95–99% pure. B cells were purified by negative selection of contaminating leukocytes and were 90–95% pure. BM-DCs were generated in RPMI 1640 medium containing 10% FCS, 2 mM glutamine, 50 μM 2-mercaptoethanol, 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and GM-CSF (around 1 μg ml^{-1}). CD40L-expressing and control NIH 3T3 fibroblast cell lines were a gift from P. Hwu.

Cytokine induction

For transfection of viral dsRNA or poly(I:C), cells were seeded in triplicate in 96-well plates at a density of 0.5–2.5 × 10^5 cells per well in 75 μl RPMI 1640 medium without FCS or other additives; where indicated, cells were seeded onto a subconfluent monolayer of CD40L-expressing or control fibroblasts or were cultured with soluble CD40L (Fc–CD40L or CD40L trimer) to amplify the cytokine response[12]. Twenty-five microlitres of transfection mix consisting of dsRNA with or without lipofectamine (GIBCO) was added to each well, and cells were incubated for 4 h. Then 100 μl complete medium was added per well and cells were cultured overnight. For electroporation, 4 × 10^6 cells were re-suspended in 200 μl 'intracellular' buffer[30] with or without dsRNA and were pulsed once at 300 V, 150 μF (Bio-Rad Gene Pulser II). Cell suspensions were then diluted with complete medium and seeded at a concentration of 2 × 10^5 cells per well in 96-well plates with or without soluble CD40L or CD40L-expressing/control fibroblasts and were cultured overnight. Supernatant was collected at 18–20 h and assayed for IL-12 p70 and IFN-α by standard sandwich enzyme-linked immunosorbent assay (ELISA) as described[12,27].

Virus infection

Influenza A/PR/8/34 virus and ΔNS1 virus[6] were used for *in vitro* infection of C57BL/6 BM-DCs. 1 × 10^6 BM-DCs per ml were infected with virus at a multiplicity of infection of 0.7, and cell supernatants and cells were collected after 8–20 h culture. IFN-α accumulation in the supernatant was measured by ELISA as described above. Infections with LCMV were performed with Armstrong 53b strain (Armstrong) or clone 13. Mice were infected intravenously with 2 × 10^6 plaque-forming units per mouse in 200 μl volume. Splenic dendritic cells were purified 3 days after infection and cultured *in vitro* at 2 × 10^6 cells per ml in 200 μl complete medium with 20 ng ml^{-1} GM-CSF. IFN-α accumulation in the supernatant after 18–20 h culture was measured using an IFN-α ELISA kit from PBL (Alexis Corporation).

Flow cytometry

For intracellular IFN-α staining, dendritic cells were electroporated with poly(I:C) and cultured for 3 h. Brefeldin A (Sigma; 5 μg ml^{-1}) was added for an additional 3 h and cells were collected and fixed in paraformaldehyde. Staining was performed in saponin-containing buffer using a mixture of rat anti-mouse IFN-α antibodies (clone F18, Hycult Biotechnology b.v. and clone RMMA-1, PBL Biomedical Laboratories), followed by biotinylated mouse anti-rat IgG (Jackson ImmunoResearch) and Streptavidin-PE (PharMingen). Cells were subsequently stained for CD11c (clone HL3, PharMingen). For intracellular staining of influenza NP and NS1 proteins, infected dendritic cells were fixed and stained as above using mouse anti-NP antibody or mouse anti-NS1 antibody followed by biotinylated rat anti-mouse IgG (Jackson ImmunoResearch) and Streptavidin-PE. Cells were subsequently stained for CD11c.

PCR

BM-DCs or CD8α$^+$ dendritic cells were analysed for TLR3 mRNA expression as described[29].

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