

# Dendritic cells require a systemic type I interferon response to mature and induce CD4<sup>+</sup> Th1 immunity with poly IC as adjuvant

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Relative to several other toll-like receptor (TLR) agonists, we found polyinosinic:polycytidylic acid (poly IC) to be the most effective adjuvant for Th1 CD4<sup>+</sup> T cell responses to a dendritic cell (DC)-targeted HIV gag protein vaccine in mice. To identify mechanisms for adjuvant action in the intact animal and the polyclonal T cell repertoire, we found poly IC to be the most effective inducer of type I interferon (IFN), which was produced by DEC-205<sup>+</sup> DCs, monocytes, and stromal cells. Antibody blocking or deletion of type I IFN receptor showed that IFN was essential for DC maturation and development of CD4<sup>+</sup> immunity. The IFN-AR receptor was directly required for DCs to respond to poly IC. STAT 1 was also essential, in keeping with the type I IFN requirement, but not type II IFN or IL-12 p40. Induction of type I IFN was mda5 dependent, but DCs additionally used TLR3. In bone marrow chimeras, radioresistant and, likely, nonhematopoietic cells were the main source of IFN, but mda5 was required in both marrow-derived and radioresistant host cells for adaptive responses. Therefore, the adjuvant action of poly IC requires a widespread innate type I IFN response that directly links antigen presentation by DCs to adaptive immunity.

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Abbreviations used: cDC, conventional DC; poly IC, polyinosinic:polycytidylic acid; PRR, pattern recognition receptor; TLRs, Toll-like receptor.

Vaccines induce protective immunity against numerous infectious diseases. However, current vaccines have limited efficacy against challenging infections like tuberculosis, malaria, and HIV, where Th1-type T cell immunity likely has a potentially important role (Seder and Mascola, 2003). Proteins represent a potential route to safe new generation vaccines (Ulmer et al., 2006), but, typically, these are poorly immunogenic for T cells when administered alone. Therefore, special attention is being given to additional adjuvants or enhancers of immunity. Adjuvants activate innate immunity and, in particular, the maturation of antigen-presenting immunostimulatory DCs (Pulendran and Ahmed, 2006; Kool et al., 2008). To understand adjuvant action, and to identify optimal ones, it is necessary to move beyond the current focus on in vitro assays and selected monoclonal or TCR transgenic T cells to the intact animal and patient. This avenue of research should also help to identify mechanisms that link innate responses to adaptive immunity in vivo.

Adjuvants can stimulate innate immunity by interacting with specialized pattern recognition receptors (PRRs), including Toll-like receptors (TLRs; Akira et al., 2006; Kawai and Akira, 2009) and nucleotide-binding oligomerization domain receptors (Li et al., 2008). DCs express a repertoire of PRR, allowing the recognition of a range of pathogen constituents. The engagement of PRR on DCs leads to de novo transcription and secretion of cytokines and chemokines, enhanced antigen presentation capacity, and migration to lymphoid tissues where the DCs interact with T cells and B cells to initiate and shape the adaptive immune response. The type of stimulus conditions DCs to adopt a Th1- or Th2-polarizing function (Mazzoni and Segal, 2004; Kwissa et al., 2007). However, many cell types, including

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nonhematopoietic cells, express PRR and produce cytokines during innate immunity (Nolte et al., 2007). To understand adjuvant action, it will likely be necessary to understand the contribution of cytokines and different cellular sources of cytokines to the initiation of immunity from the naive polyclonal repertoire.

Among innate inflammatory cytokines, type I IFNs are considered to be major players for linking innate to adaptive immunity. First uncovered for their innate antiviral activity (Nagano and Kojima, 1954; Lindenmann et al., 1957), type I IFNs also enhance adaptive responses. They activate DCs, critical antigen-presenting cells for initiating immunity (Blanco et al., 2001), by promoting the expression of costimulatory molecules (Luft et al., 1998; Gallucci et al., 1999; Ito et al., 2001). This includes human blood monocytes, where type I IFN can stimulate differentiation into DCs (Paquette et al., 1998; Santini et al., 2000). Type I IFN-treated DCs prime T cells *in vitro* more effectively. The DCs also up-regulate expression of lymph node-homing CCR7 and exhibit stronger migratory capacity compared with DCs differentiated with other cytokines (Paquette et al., 1998; Parlato et al., 2001). Type I IFN further promotes cross-priming of CD8<sup>+</sup> T cells by direct stimulation of DCs (Le Bon et al., 2003). Le Bon et al. (2001) showed that type I IFNs act on *ex vivo*-derived DCs to become better stimulators of antibody immunity. Nevertheless, the effects of type I IFN on DCs *in situ* need definition to understand the link between the innate production of cytokines to adaptive immunity.

Type I IFNs also have direct effects on T cells, particularly CD8<sup>+</sup> T cells (Tough et al., 1996), by extending their survival during antigen-driven clonal expansion (Kolumam et al., 2005; Le Bon et al., 2006). In contrast, the importance of type I IFNs in the generation of antigen-specific CD4<sup>+</sup> T cell responses is uncertain. Type I IFN inhibits secretion of Th2 cytokines (IL-4 and IL-5) and stimulates type II IFN or IFN- $\gamma$  production (Brinkmann et al., 1993; Wenner et al., 1996). Type I IFNs inhibit the death of activated CD4<sup>+</sup> T cells (Marrack et al., 1999), a finding which was later extended to the adoptive transfer of antigen-specific CD4<sup>+</sup> T cells lacking the expression of type I IFN receptors (Havenar-Daughton et al., 2006). However, although it was originally believed to induce STAT4 activation, type I IFN failed to promote Th1 commitment in human and mouse CD4<sup>+</sup> T cells *in vitro*. Despite induction of detectable STAT4 tyrosine phosphorylation, the activation was transient and insufficient to drive Th1 development in the absence of IL-12 (Berenson et al., 2004; Ramos et al., 2007). Therefore, the effect of type I IFNs on the generation of antigen-specific CD4<sup>+</sup> T cells *in vivo* is unclear from these observations.

Several TLR agonists are currently being evaluated as adjuvants for vaccine development. Among them, synthetic double-stranded RNA, polyinosinic:polycytidylic acid (poly IC), induces inflammation and long-lasting T cell immunity (Salem et al., 2006; Trumpfheller et al., 2008; Stahl-Hennig et al., 2009). Poly IC is recognized by TLR3 located mostly in endosomal membranes. Also, poly IC binds the cytoplasmic RNA

helicase MDA5, resulting in activation of IRF-3 and representing a major pathway for the production of type I IFNs in a TLR-independent fashion (Kato et al., 2006). Poly IC is a strong inducer of type I IFN. Although DCs and macrophages produce type I IFN upon poly IC stimulation *in vitro*, many nonhematopoietic cell types potentially could provide a source of this cytokine *in vivo* (Alexopoulou et al., 2001; Gitlin et al., 2006). In addition, the critical question of whether type I IFN is required for the adjuvant effect of poly IC and its mechanism of action *in situ* has not been addressed.

In this paper, we study the mechanism whereby poly IC adjuvants CD4<sup>+</sup> Th1 immunity for an HIV gag protein vaccine that is targeted *in vivo* to DCs within a monoclonal antibody to the uptake receptor DEC-205/CD205 (Trumpfheller et al., 2008). We found that poly IC is a superior adjuvant and then we made several findings on its effects *in vivo*. First, type I IFN is the dominant driver of two innate cellular responses: the maturation of DCs and the activation of NK cells to produce type II IFN. Second, type I IFN must act directly on DCs to induce their maturation into immunostimulatory cells and also is necessary for the generation of a Th1 CD4<sup>+</sup> adaptive T cell response, whereas IL-12 and type II IFN-R are not. Third, both bone marrow-derived and stromal cells play essential roles in providing type I IFN. These data illustrate that poly IC adjuvants Th1 CD4<sup>+</sup> T cell immunity by replicating the early systemic effects of viral infection through its superior and widespread induction of type I IFN.

## RESULTS

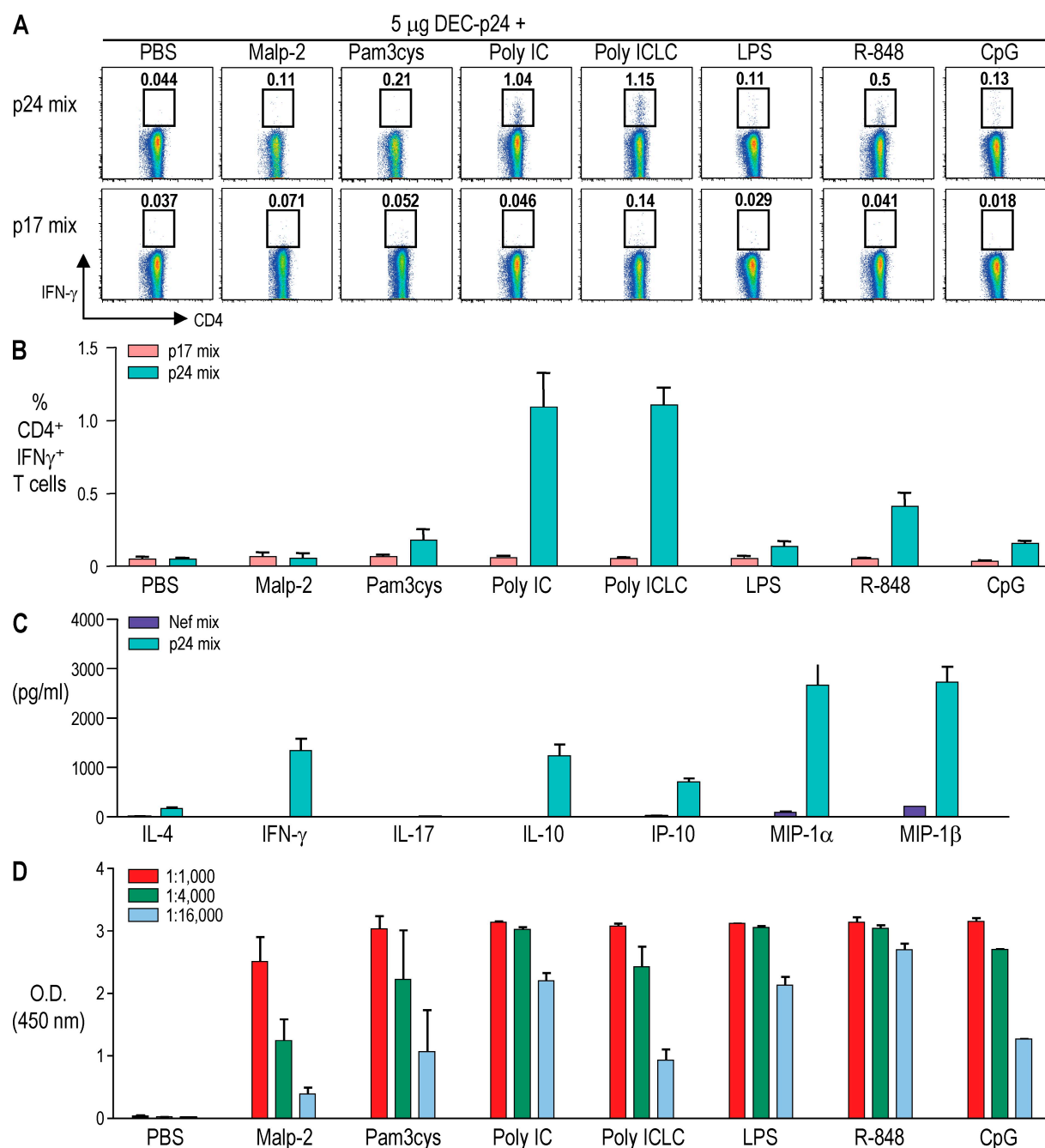
### Poly IC is a superior adjuvant for CD4<sup>+</sup> T cell responses to a protein vaccine

We had previously shown that poly IC was a superior adjuvant to monophosphoryl lipid A for inducing strong protective CD4<sup>+</sup> T cell responses to HIV gag p24 delivered within an anti-DEC antibody (Trumpfheller et al., 2008). To find the most appropriate adjuvant for this new approach to protein-based vaccines, we extended our analysis to other TLR ligands, including MALP-2 (TLR2/TLR6), Pam3Cys (TLR1/TLR2), poly IC (TLR3), poly ICLC (TLR3-complexed poly IC with carboxymethylcellulose and poly-L-lysine to improve resistance to ribonucleases), LPS (TLR4), R-848 (TLR7/TLR8) delivered as an aqueous compound or within a topical cream, and CpG (TLR9). Mice were immunized with  $\alpha$ -DEC-gag p24 mAb and specific adjuvants twice *i.p.* over 4 wk. 1 wk after the final injection, antigen-specific responses were evaluated by IFN- $\gamma$  secretion in response to gag p24 peptides, primarily by multicolor flow cytometry.

Poly IC, together with its analogue poly ICLC, induced stronger gag-specific CD4<sup>+</sup> T cell responses compared with the other adjuvants (Fig. 1, A and B) with frequencies of >1% of IFN- $\gamma$  producing CD3<sup>+</sup>CD4<sup>+</sup> T cells after *i.p.* injection. The improved CD4<sup>+</sup> T cell responses with poly IC and poly ICLC were also evident at the single cell level when IL-2 and TNF- $\alpha$  were measured (Fig. S1). ELISA assays showed that IL-4 and IL-17 were not induced during the adjuvant role of

poly IC or poly ICLC, verifying a polarized Th1 response (Fig. 1 C). The second most active adjuvant was R-848 applied in a cream to the skin, but the other adjuvants were

weak (Fig. 1, A and B). To verify the activity of the adjuvants, we also measured antibody responses to HIV gag and found that all the adjuvants were active, with LPS being at least as



**Figure 1. Poly IC is a superior adjuvant to elicit CD4<sup>+</sup> T cell immunity.** (A) Cx66 F<sub>1</sub> mice were primed and boosted 4 wk apart with 5  $\mu$ g  $\alpha$ -DEC-p24 and 50  $\mu$ g poly IC, 50  $\mu$ g Pam3cys, 0.5  $\mu$ g Malp2, 50  $\mu$ g poly ICLC, 10  $\mu$ g LPS, 50  $\mu$ g CpG, or R-848 cream. R-848 cream was applied topically over 5 cm<sup>2</sup> on the back of shaved mice. IFN- $\gamma$  secretion in gated CD3<sup>+</sup>CD4<sup>+</sup> splenic T cells in response to HIV gag p24 peptides was measured 1 wk after boost. (B) As in A, but means  $\pm$  SD from five (poly IC), three (poly ICLC, R-848, and CpG), and two (Malp-2, Pam3cys, and LPS) experiments, with three mice per group. (C) Cx66 F<sub>1</sub> mice were primed and boosted 4 wk apart with 5  $\mu$ g  $\alpha$ -DEC-p24 and 50  $\mu$ g poly IC. 1 wk after boost, bulk splenocytes were stimulated with gag p24 peptide mix or control peptide mix for 3 d. The concentrations of the indicated cytokines and chemokines were measured in cell culture supernatants by luminex. Samples were acquired in duplicate and analyzed by ELISA. Bars represent the mean  $\pm$  SD from two experiments (six mice total). (D) Mice were immunized as in A. 1 wk after boost, gag-specific antibodies in serum samples were measured by ELISA. Data are given as OD mean  $\pm$  SD from two independent experiments (five mice total).

active as poly IC (Fig. 1 D). These results encouraged us to compare the innate response to poly IC relative to other agonists to help explain its superior adjuvant role in inducing Th1 type CD4<sup>+</sup> T cell immunity.

### The innate response to poly IC uniquely leads to high levels of serum type I IFN

To assess the innate response to poly IC and poly ICLC, we studied the systemic release of inflammatory cytokines, a prototype response to agonists for PRR. We did ELISA assays on serum at 1, 3, 6, or 20 h after administration of different TLR ligands *i.p.*, measuring IL-6, TNF- $\alpha$ , IL-12p40, IFN- $\gamma$ , IFN- $\alpha$ , and IFN- $\beta$ . All TLR agonists, to different extents, induced production of IL-6, TNF- $\alpha$ , and IL-12 p40 (Fig. 2), whereas serum IL-12p70, IL-1 $\beta$ , and IL-10 were not detected (not depicted). Interestingly, production of type I and type II IFNs was more restricted, being detected primarily with poly IC, poly ICLC, and R-848. Detection of IFN- $\alpha$  and IFN- $\beta$  was transient, peaking at 3–6 h, and was highest with both poly IC and poly ICLC (Fig. 2). Thus, strong type I IFN production is a special feature of poly IC, suggesting a role for IFN in its adjuvant effect.

### Poly IC induces activation of DCs

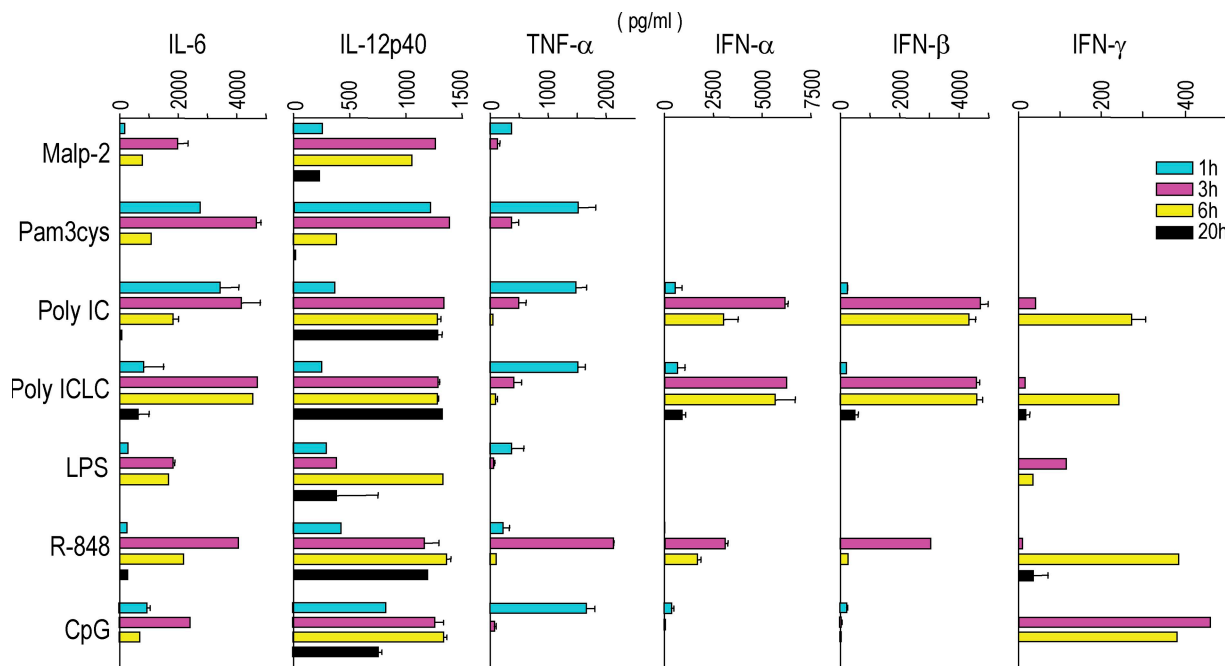
Poly IC matures both mouse and human DCs (Verdijk et al., 1999; Blanco et al., 2001; Lopez et al., 2004; Tsujimoto, 2006), and this maturation was shown to depend on type I IFN (Honda et al., 2003). However, these experiments were performed *in vitro*. To assess DC maturation *in vivo*, mice were injected with 50  $\mu$ g poly IC, and the effect of type I IFN was

blocked by simultaneous injection of IFNAR1 (anti-IFN- $\alpha\beta$  receptor) antibody or isotype control. After 12 h, expression of MHC-II and costimulatory molecules (CD40 and CD86) on DEC<sup>+</sup> and DEC<sup>-</sup> DCs was analyzed by flow cytometry (Fig. 3 A). Blocking type I IFN receptor effectively reduced DC maturation, as monitored by the up-regulation of these immunostimulatory molecules in response to poly IC.

To directly evaluate the stimulatory capacity of DCs after poly IC stimulation with or without blockade of type I IFN receptors, we isolated the CD11c<sup>+</sup> DC fraction from the spleen, fixed the cells with formaldehyde to block further differentiation in culture, and added the DCs in graded doses to allogeneic T cells in an MLR. DCs from poly IC-treated mice became active stimulators of the allogeneic MLR, whereas blockade of type I IFNs abrogated this effect (Fig. 3 B), showing that this cytokine was essential for DC maturation in response to the poly IC adjuvant.

### Direct IFNAR signaling is critical for DC activation *in vivo*

To determine if type I IFN had to act directly on DCs to bring about maturation, we made mixed bone marrow chimeras in which the injected bone marrow suspension was a 50:50 mixture of CD45.1 WT and CD45.2 IFN-AR<sup>-/-</sup> cells (Fig. 4 A). When poly IC was given to these chimeric mice, only the CD45.1 WT cells were able to up-regulate costimulatory molecules and MHC class II (Fig. 4 B) and exhibit enhanced MLR stimulatory activity (Fig. 4 C and Fig. S3). Therefore, Type I IFN directly promotes maturation of DCs to link innate to adaptive immunity.



**Figure 2. Induction of inflammatory cytokines in the serum by TLR ligands.** Mice were treated *i.p.* with 50  $\mu$ g poly IC, 50  $\mu$ g Pam3cys, 0.5  $\mu$ g Malp2, 10  $\mu$ g LPS, 50  $\mu$ g poly ICLC, 50  $\mu$ g CpG, and 5 cm<sup>2</sup> of 0.2% R-848 cream. IL-6, TNF- $\alpha$ , IL-12p40, IFN- $\alpha$ , IFN- $\beta$ , and IFN- $\gamma$  were analyzed in serum at different time points (means of four mice). Error bars indicate the mean  $\pm$  SD. Data are representative of two similar independent experiments.

### Poly IC induces IFN- $\gamma$ production by NK cells

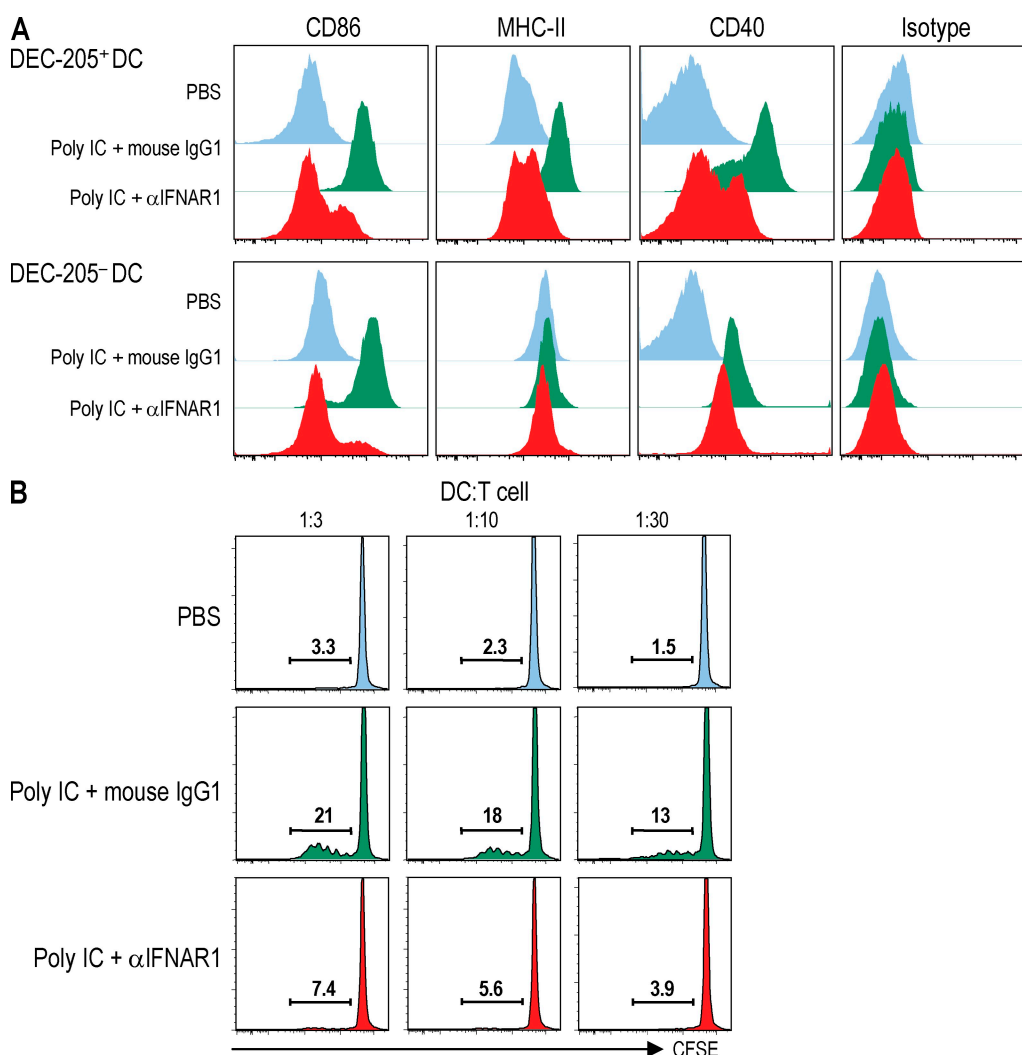
An additional component to the innate response to the *in vivo* administration of poly IC is the rapid activation of NK cells to produce IFN- $\gamma$  (Salem et al., 2006). Because we had observed an innate IFN- $\gamma$  response to poly IC, we assessed NK cells as a possible source. We found that CD11c<sup>int</sup>DX5<sup>+</sup> NK cells were major producers of IFN- $\gamma$ , whereas other cells, including CD11c<sup>high</sup>DX5<sup>-</sup> DCs were inactive (Fig. 5, A and B).

Human NK cells respond directly to poly IC and CpG to produce IFN- $\gamma$  (Lauzon et al., 2006) but, in contrast, purified mouse NK cells fail to respond directly to TLR agonists and require the presence of DCs or IL-12 (Sawaki et al., 2007). To assess the latter possibility, highly purified splenic NK cells were incubated overnight in the presence of poly IC, and IFN- $\gamma$  production was measured by ELISA. As a positive control, the NK cells were stimulated with rIL-12 and rIL-15

(Walzer et al., 2005). In concordance with previous studies, poly IC failed to directly activate mouse NK cells (Fig. 5 C). We then considered that NK activation was indirect and a result of stimulation by type I IFNs. To test this, mice were injected with poly IC 1 h after injection of anti-IFNAR1 or isotype control. Antibody treatment dramatically reduced IFN- $\gamma$  production by NK cells (Fig. 5 D). Collectively, these results highlight the importance of type I IFN during the innate responses of both DCs and NK cells in the intact animal.

### Poly IC directly activates multiple cell types to produce type I IFN

Type I IFNs are rapidly produced by many cell types in response to immune and/or inflammatory stimuli. Poly IC was previously shown to stimulate mouse conventional DCs (cDCs), bone marrow-derived DCs, and macrophages to produce



**Figure 3. Type I IFN signaling controls DC maturation.** (A) 1 h after 100  $\mu$ g of anti-IFAR1 blocking antibody or isotype control, mice were stimulated with 50  $\mu$ g poly IC for 12 h and analyzed for cell surface MHC-II, CD86, and CD40 on DEC<sup>+</sup> and DEC<sup>-</sup> DCs. (B) As in A, but graded numbers of fixed splenic DCs from BALB/C mice were added for 5 d to  $2 \times 10^5$  allogeneic C57BL/6 T cells. T cell proliferation was detected by CFSE dilution of CD3<sup>+</sup> cells. Data are representative of three experiments.

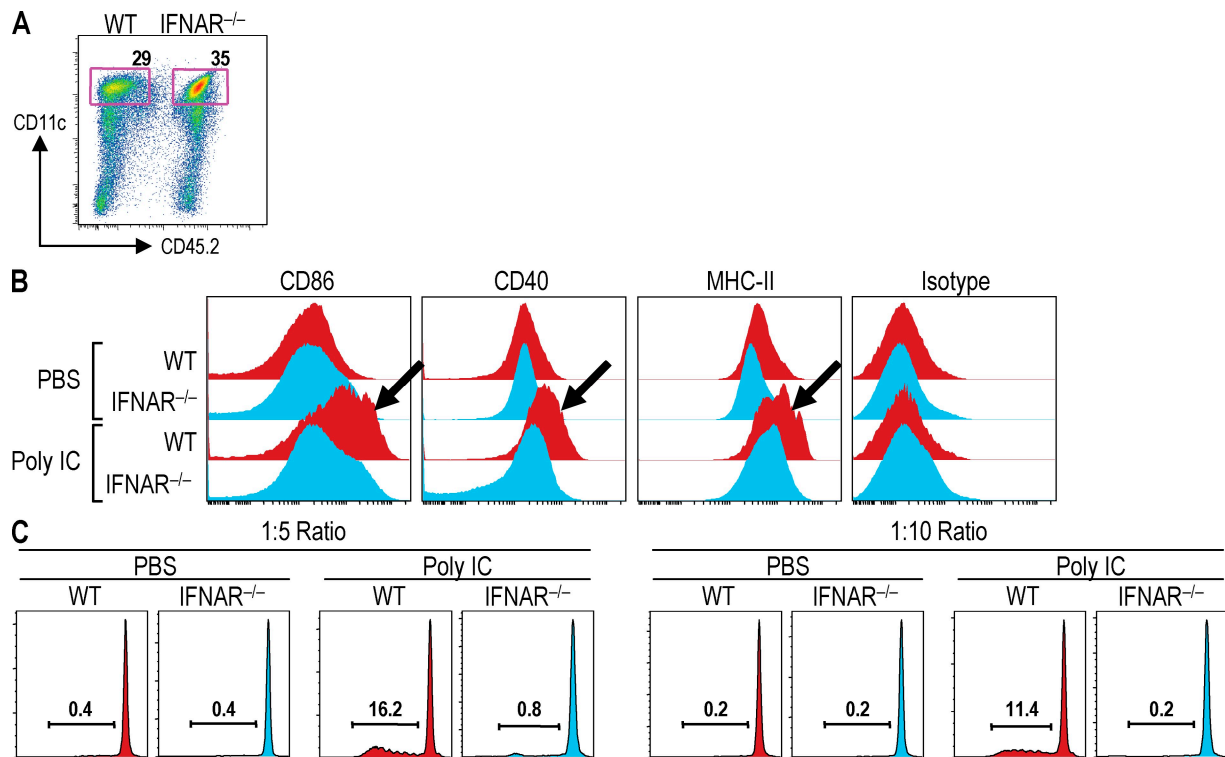


type I IFN (Gitlin et al., 2006; Kumagai et al., 2007). Splenic cells were sorted into several different cell types, stimulated in vitro with poly IC, and tested for the production of type I IFN. First, spleen cells were pre-enriched by magnetic depletion of B, T, and erythroid cells with antibodies to CD19, CD3, and TER119. The depleted cells were then sorted based on the expression of a set of specific markers. NK cells were  $CD3^{-}DX5^{+}$ , plasmacytoid DCs (pDCs) were  $CD11c^{int}DX5^{-}B220^{+}$ , and cDCs were  $CD11c^{hi}DX5^{-}CD8^{-}$  or  $CD8^{+}DEC-205^{+}$  (Fig. 6 A; Asselin-Paturel et al., 2005; Blasius et al., 2007; Dudziak et al., 2007). To sort granulocytes and monocytes, we gated for  $CD11b^{hi}$  cells and then isolated  $LyC6^{hi}$  granulocytes and  $CD115^{+}$  monocytes. Macrophages were defined as  $CD11b^{low}F4/80^{+}$  cells (Fig. 6 A; Taylor et al., 2005; Randolph et al., 2008).

When each of the sorted cell subsets ( $3 \times 10^5$  cells/well) were stimulated overnight with poly IC, both  $CD8^{+}DEC^{+}$  DCs and monocytes produced type I IFN, whereas  $CD8^{-}$  DCs, pDCs, NK, granulocytes, and macrophages did not (Fig. 6 B). To test the capacity of nonhematopoietic cells to produce IFN- $\beta$ , we used splenic stromal cells ( $10^5$  cells/well) that had been expanded from CD45-negative spleen cells. Similar to monocytes and  $DEC^{+}$  DCs, stromal cells were able to produce type I IFN (Fig. 6 B).

Although production of type I IFN by pDCs requires activation of the TLR system, cDCs and fibroblasts were shown to secrete type I IFNs in response to viral infection in a cytosolic helicase-dependent fashion (Kato et al., 2005). Because poly IC can be recognized by the RNA helicase mda5, we next tested if poly IC-induced IFN- $\beta$  required this PRR. Monocytes,  $DEC^{+}$  DC, and stromal cells from WT,  $mda5^{-/-}$ , or  $TLR3^{-/-}$  mice were stimulated in vitro with poly IC as described in the previous paragraph. Interestingly, the production of IFN- $\beta$  by all cell types was comparable between WT and  $TLR3^{-/-}$  cells, whereas it was severely impaired in  $mda5^{-/-}$  cells (Fig. 6 C). Similar results were obtained when we measured the release of type I IFN into the serum of mice genetically deficient in these PRRs after poly IC injection (Fig. S2), confirming prior observations (Gitlin et al., 2006).  $DEC^{+}$  DCs were also able to produce small amounts of IFN- $\beta$  in an  $mda5$ -independent fashion (Fig. 6 C), but this was ablated in mice doubly deficient in TLR3 and  $mda5$  (not depicted).

To investigate the extent to which type I IFN in vivo required hematopoietic cells, e.g.,  $DEC^{+}$  DCs and monocytes, and nonhematopoietic cells, e.g., endothelia, stromal bone marrow chimeras were generated by reconstituting  $mda5^{-/-}$  mice with WT bone marrow or, alternatively, WT mice



**Figure 4. Direct type I IFN signaling of DCs is required for their maturation.** Mixed bone marrow chimeras were prepared with a 50:50 mixture of bone marrow from WT ( $CD45.2^{-}$ ) and  $IFNAR^{-/-}$  ( $CD45.2^{+}$ ) mice. (A) Gating strategy for WT ( $CD45.2^{-}$ ) and  $IFNAR^{-/-}$  ( $CD45.2^{+}$ ) DCs 4–6 wk after chimerism. (B) The chimeras were stimulated with 50  $\mu$ g poly IC or PBS for 12 h. Expression of CD86, MHC-II, and CD40 on WT and  $IFNAR^{-/-}$  DCs, was analyzed by FACS. Arrows show increased up-regulation of costimulatory molecules in  $IFNAR^{-/-}$  DCs. (C) The chimeras were stimulated as in B. After  $CD11c^{hi}$  enrichment, WT and  $IFNAR^{-/-}$   $CD11c^{hi}$  were FACS sorted as in A. Graded numbers of fixed B6.WT or B6. $IFNAR^{-/-}$  splenic DCs were added for 5 d to  $2 \times 10^5$  allogeneic Balb/C T cells. T cell proliferation was detected by CFSE dilution of  $CD3^{+}$  cells. Cells numbers are indicated as  $10^3$ . Data are representative of two similar independent experiments.

were reconstituted with *mda5*<sup>-/-</sup> marrow. The different chimeras were injected i.p. with poly IC, and serum was collected after 6 h. Interestingly, WT recipients of *mda5*<sup>-/-</sup> marrow produced high levels of IFN- $\alpha/\beta$ , whereas *mda5*<sup>-/-</sup> recipients of WT marrow were severely compromised (Fig. 6 D). These data demonstrated that stimulation of radioreistant cells, but not bone marrow-derived cells, was required for the bulk of type I IFN production after poly IC treatment.

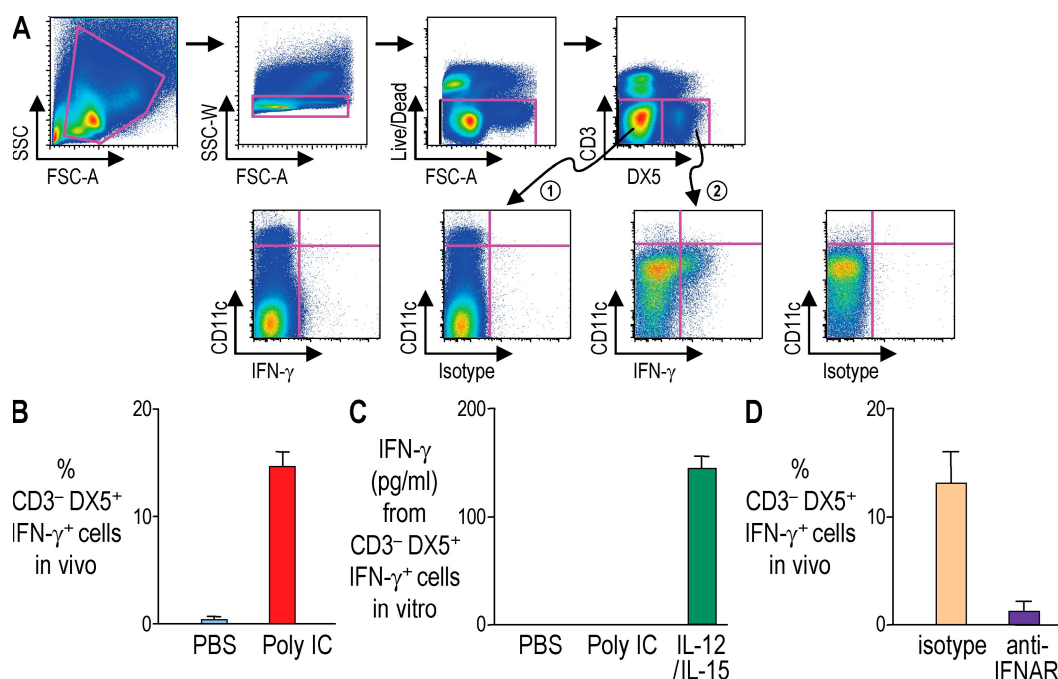
### Production of type I IFNs by poly IC, but not IL-12 p40, is needed for Th1 immunity

To determine if type I IFN could influence the development of adaptive Th1 immunity, we first analyzed gag-specific CD4<sup>+</sup> T cells responses after IFNAR1 blocking of mice immunized with either  $\alpha$ -DEC-p24 or nontargeted gag p41 protein. In both cases, the adjuvant effect of poly IC was markedly reduced by IFNAR1 blocking compared with isotype control (Fig. 7 A and Fig. S4 A). TNF- $\alpha$  and IL-2 production likewise was also severely reduced (unpublished data). Similar results were obtained with IFNAR<sup>-/-</sup> mice, which were unable to develop adaptive Th1 immunity in response to  $\alpha$ -DEC-p24 plus poly IC (Fig. 7 B). To extend the evidence for a critical role of type I IFNs, we studied mice with a genetic deletion of STAT1, the major signal transducer for type I IFNs. As anticipated, immunization with either  $\alpha$ -DEC-p24 or p41 protein was totally ablated in STAT1<sup>-/-</sup> mice (Fig. 7 C and Fig. S4 B). Thus, the stimulation of type I IFNs by poly IC is required for its adjuvant role.

Type I IFNs may regulate Th1 responses by indirect mechanisms. IFN- $\alpha\beta$  signaling can up-regulate IFN- $\gamma$  production by DCs, promoting the ability of naive T cells to respond to IL-12 (Wenner et al., 1996; Montoya et al., 2002), whereas type I IFN can up-regulate expression of IL-12R $\beta$ 2 on human CD4<sup>+</sup> T cells, increasing responsiveness to IL-12, a pivotal cytokine for Th1 responses (Rogge et al., 1997). To determine if type I IFN might additionally require either increased IFN- $\gamma$  or IL-12 signaling, CD4<sup>+</sup> T cell responses to DEC-p24 and poly IC were analyzed in IFN- $\gamma$ R (IFN- $\gamma$  receptor) and IL-12 p40-deficient mice. Both IFN- $\gamma$ R<sup>-/-</sup> and IL-12p40<sup>-/-</sup> mice mounted similar responses to WT mice (Fig. 7, D and E and Fig. S4, C and D), implying that type I IFN, but not type II IFN or IL-12, was directly required for poly IC to induce polarized Th1 immunity.

### IFN from both bone marrow-derived and nonhematopoietic cells is needed for the adjuvant function of poly IC

Because *mda5* and TLR3 are the major PRRs for type I IFN production in response to poly IC, and both receptors were needed to induce gag-specific CD4<sup>+</sup> T cell responses (Trumpfheller et al., 2008), we analyzed chimeric mice in which either the bone marrow donor or recipient were lacking both *mda5* and TLR3. The B and T cells in these mice were >98% donor in origin after typing with anti-CD45.1 and anti-CD45.2 (unpublished data). In each case, i.e., when either the hematopoietic cells or nonhematopoietic cells could not



**Figure 5. NK cells are major producers of IFN- $\gamma$  after poly IC.** Mice were injected i.p. with 50  $\mu$ g poly-IC or PBS. After 2 h, spleens were collected and a single cell suspension of splenocytes was incubated with 10  $\mu$ g/ml BFA for 3 h before intracellular staining for IFN- $\gamma$ . (A) Five color flow cytometry to identify IFN- $\gamma$ -positive splenocytes. (B) As in A, but production of IFN- $\gamma$  by NK cells gated as CD3<sup>-</sup> DX5<sup>+</sup> cells. Means  $\pm$  SD of four experiments. (C) Highly purified CD3<sup>-</sup> DX5<sup>+</sup> NK cells.  $3 \times 10^5$  cells/well were stimulated with 50  $\mu$ g/ml poly IC or, as a positive control, a combination of rIL-12 and rIL-15. After 12 h, IFN- $\gamma$  in culture supernatants was detected by ELISA. Means  $\pm$  SD of three independent experiments are shown. (D) Mice were injected with poly IC and anti-IFNAR1 blocking antibody or isotype control. Production of IFN- $\gamma$  was detected by intracellular staining. Mean  $\pm$  SD from three experiments is shown.

make type I IFN via poly IC, its adjuvant effect was reduced by >80% (Fig. 8). These results indicate that a systemic innate response, and not only a local response by antigen-capturing DCs, accounts for the adjuvant role of poly IC.

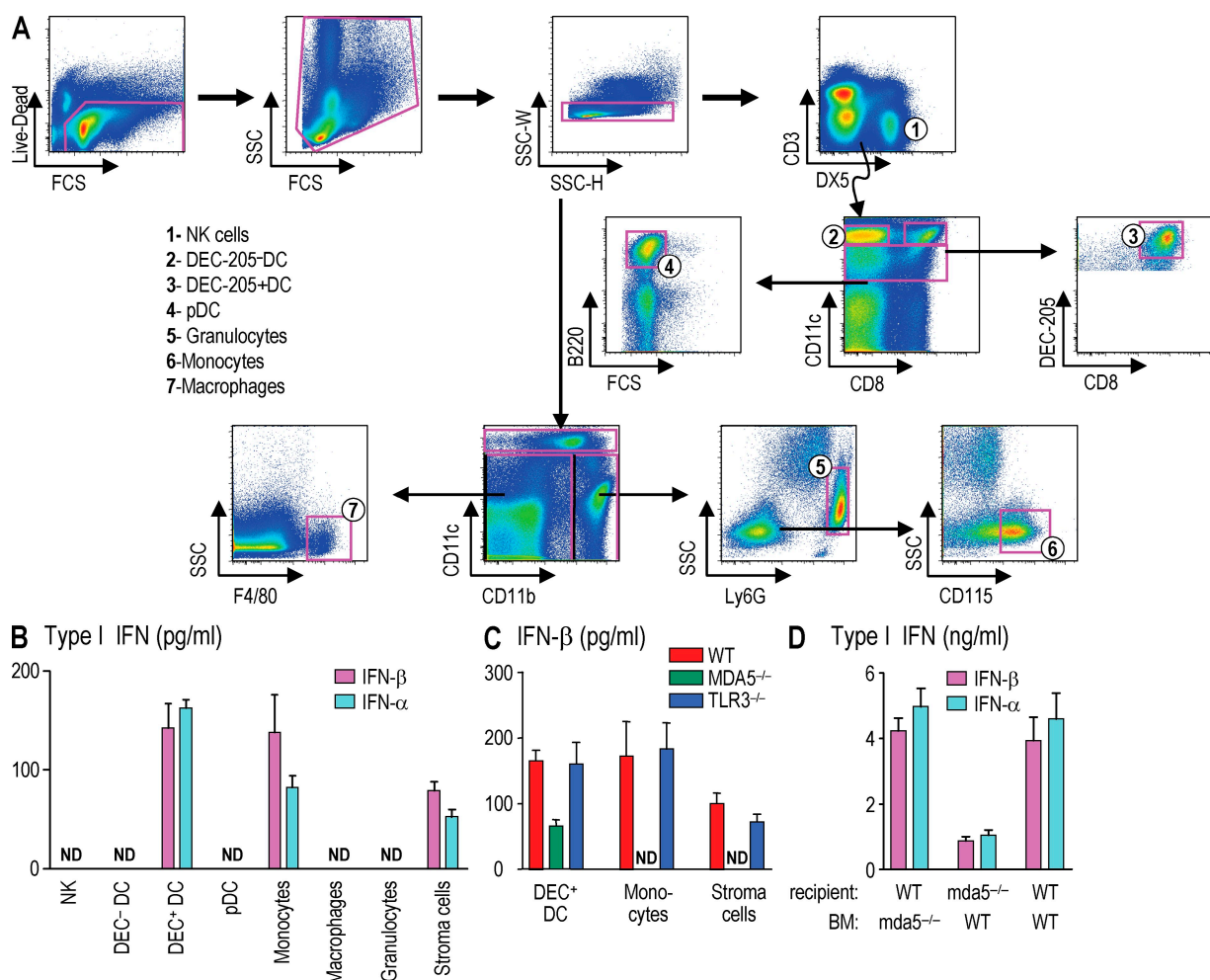
## DISCUSSION

### Poly IC as a clinically feasible adjuvant for protein-based vaccines

Vaccines, particularly those that elicit protective T cell immunity, are comprised of antigens and adjuvants, with the latter influencing the quantity and quality of the specific immune response. Vaccine science can be extended by understanding the mechanism of adjuvant action, particularly in the setting of the intact

animal and a polyclonal T cell repertoire. In this paper, we have analyzed the adjuvant mechanism of poly IC in an experimental system in which HIV gag is the antigen and the elicited immune response is durable, broad (many peptides recognized), and capable of providing protective immunity (Trumpfheller et al., 2008). In contrast to the findings in the current paper, many prior studies of adjuvants, like CpG and LPS, have analyzed isolated cells and the clonal expansion of TCR transgenic T cells (Lore et al., 2003; Napolitani et al., 2005; Thompson et al., 2005; He et al., 2007; Mata-Haro et al., 2007).

Our interest in poly IC was initially based on two sets of findings. Poly IC has been used in humans to induce type I IFN in cancer patients and had a good safety record (Robinson



**Figure 6. Production of IFN-β by multiple cell types.** (A) Splenocytes from WT mice were depleted with Ter119<sup>-</sup>, CD3<sup>-</sup>, and CD19<sup>-</sup> biotinylated antibodies using streptavidin magnetic beads before cell sorting. For monocytes, macrophages, and granulocytes, splenocytes were additionally depleted of DX5<sup>+</sup> cells. cDCs (CD3<sup>-</sup>DX5<sup>-</sup>CD11c<sup>hi</sup>CD8<sup>-</sup> and CD8<sup>+</sup>DEC205<sup>+</sup>), pDCs (CD3<sup>-</sup>DX5<sup>-</sup>CD11c<sup>low</sup>B220<sup>+</sup>), NK cells (CD3<sup>-</sup>DX5<sup>+</sup>), macrophages (CD11b<sup>low</sup>F4/80<sup>+</sup>), monocytes (CD11b<sup>hi</sup>CD115<sup>+</sup>), and granulocytes (CD11b<sup>hi</sup>Ly6G<sup>+</sup>) were sorted based on their expression of specific markers (see text). Numbers 1–6 indicate the different cell types sorted. (B) Highly purified cells were plated at  $3 \times 10^5$  cells/well in a round-bottomed 96-well plate and incubated with 50 μg/ml poly IC or medium alone. Splenic stromal cells, obtained as described in the Materials and methods, were plated at  $5 \times 10^4$  cells/well in a 48-well plate. Supernatants were collected after 12 h and production of Type I IFN was detected by ELISA. (C) As in B, DEC<sup>+</sup> DCs, monocytes, stromal cells from WT, TLR3<sup>-/-</sup>, and mda5-deficient mice were stimulated in vitro with 50 μg/ml poly IC or medium alone. (D) WT and mda5<sup>-/-</sup> mice were lethally irradiated and injected with bone marrow cells from WT or mda5<sup>-/-</sup> mice. After 6 wk, chimeras were injected with 50 μg poly IC. Serum was collected 6 h later and analyzed by Type I IFN ELISA kit. Error bars show the means  $\pm$  SD from at least two experiments. ND, not detectable.

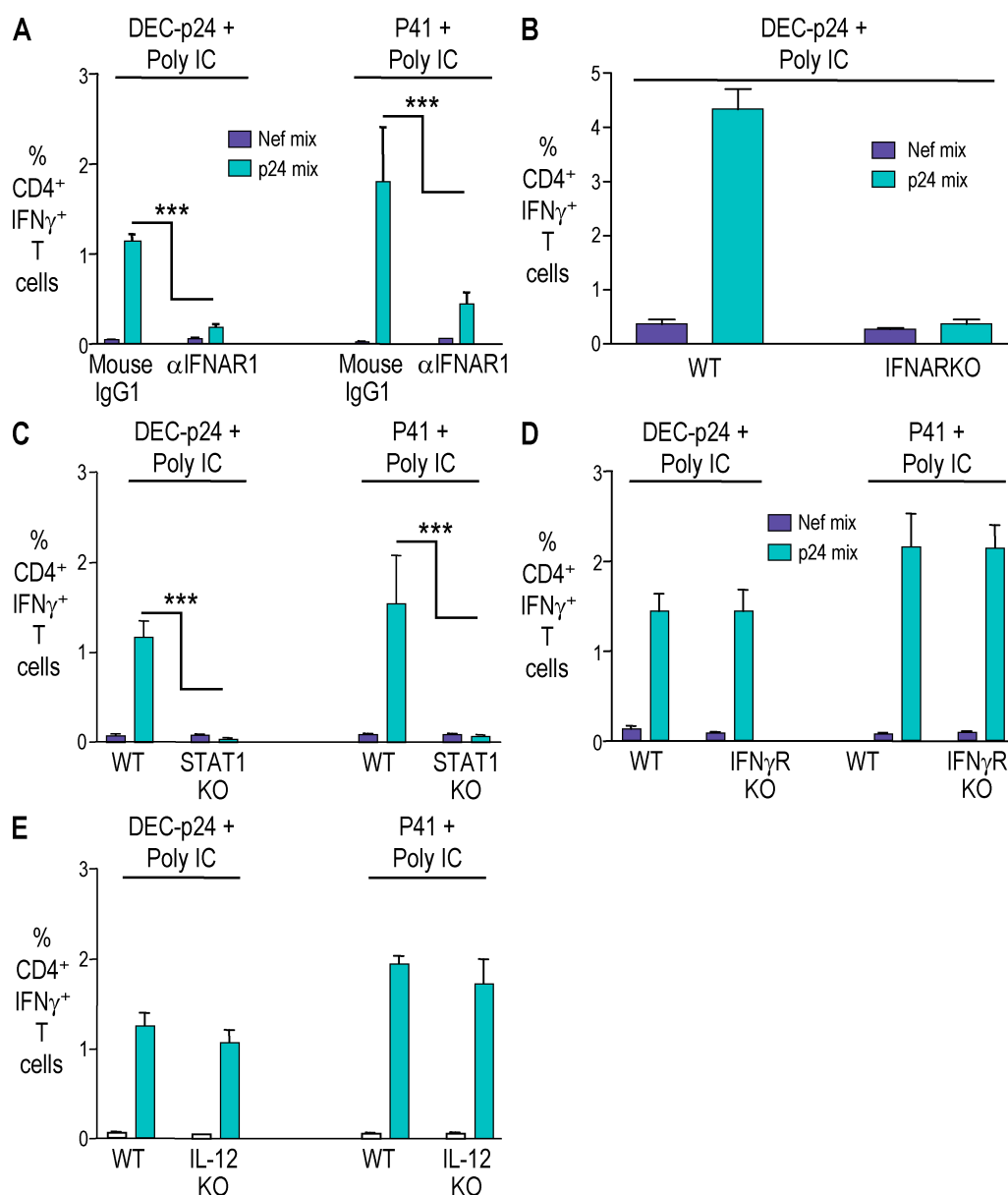


et al., 1976). These studies were directed purely to the innate IFN response, rather than adjuvanting adaptive Th1 immunity. Also, Stahl-Hennig et al. (2009) have shown that poly IC is a more potent adjuvant than other agonists for responses to protein antigens in rhesus macaques. We find that poly IC is superior to several other candidate adjuvants and have identified important mechanistic aspects. For example, although both monocytes and DEC<sup>+</sup> DCs are able to make type I IFN in response to poly IC, we found in bone marrow chimera experiments that the main source of type I IFN in

vivo after administration of poly IC emanates from non-hematopoietic radioresistant cells. We proceeded to analyze the contribution of the type I IFN response to adjuvant action.

#### Type I IFN but not type II or IL-12 p40 mediates the adjuvant action of poly IC

A recognized feature of adjuvants is the induction of inflammatory cytokines through PRR (Kool et al., 2008; Li et al., 2008). We find that one type of cytokine, type I IFN, dominated the adjuvant role of poly IC during CD4<sup>+</sup> Th1 immunity in vivo.



**Figure 7. The adjuvant role of poly IC is dependent on type I IFNs and independent of IL-12 p40.** (A) Cx36 F<sub>1</sub> mice were injected i.p. with 5  $\mu$ g  $\alpha$ -DEC-p24 or 10  $\mu$ g gag-p41 and 50  $\mu$ g poly IC together with anti-IFNAR1 or isotype control. Mice were boosted with the same conditions at 4 wk. 1 wk later, HIV gag-specific CD3<sup>+</sup> CD4<sup>+</sup> splenic T cells were analyzed for IFN- $\gamma$ . (B–E) As in A, the percentage of CD3<sup>+</sup> CD4<sup>+</sup> T cells producing IFN- $\gamma$  was measured in WT, IFNAR<sup>0/0</sup>, STAT1<sup>0/0</sup>, IFN- $\gamma$ R<sup>0/0</sup>, and IL-12 p40<sup>0/0</sup> immunized mice, respectively. Means  $\pm$  SD are shown of two independent experiments with a total of six mice. \*\*\*,  $P = 0.001$ .

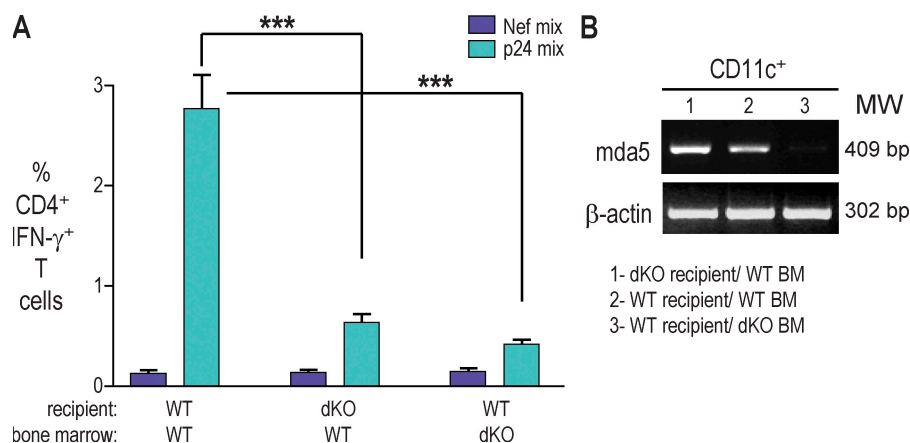
Although type I IFN has been proposed to have a role during clonal expansion and survival of CD8<sup>+</sup> T cells, its role in CD4<sup>+</sup> Th1 development was unclear (see Introduction). However, prior experiments were done *in vitro* with recombinant cytokines and using CD3 antibody stimulation or transgenic models, making it difficult to extrapolate *in vivo*. We did consider the possibility that type I IFN could act indirectly by promoting secretion of other Th1-enhancing cytokines, IL-12 and IFN- $\gamma$ . IL-12 plays a crucial role in Th1 priming (Trinchieri, 1994), whereas IFN- $\gamma$  increases responsiveness to IL-12 and, in addition, activates the Th1-specific transcription factor T-bet (Lighvani et al., 2001; Afkarian et al., 2002). Nevertheless, our findings *in vivo* show that deletion of IL-12 p40 or IFN- $\gamma$  receptor genes did not abrogate the CD4<sup>+</sup> T cell response to a protein plus poly IC vaccine, which predominantly depends on type I IFN to be an adjuvant.

The relevance of IL-12 *in vivo* seems to depend on the nature of the infecting or immunizing agent. IL-12 is a major cofactor for Th1 differentiation during infection with several bacteria and parasites (Gazzinelli et al., 1994; Cooper et al., 1997) as well as soluble antigens together with adjuvant, e.g., MPLA, Quil A, and CpG (Magram et al., 1996; Smith et al., 1999; Puggioni et al., 2005; Iborra et al., 2008). Yet for some virus infections, e.g., lymphocytic choriomeningitis virus and mouse hepatitis virus, the lack of IL-12 has little or no effect on the induction of Th1 immunity (Schijns et al., 1998; Oxenius et al., 1999). One criterion for the selection of a suitable Th1 adjuvant has been the production of IL-12 by DCs *in vitro*, in particular, when searching for synergistic effects of multiple TLR ligands (Napolitani et al., 2005). In contrast, we could not detect systemic production of IL-12 p70 after administration of poly IC, whereas poly IC was superior at inducing systemic type I IFNs. Our findings indicate that poly IC mimics infections with certain viruses,

rather than bacteria and parasites, and does not require the presence of IL-12 for the induction of Th1 immunity.

#### Type I IFN activates NK cells to produce type II IFN *in vivo*, but the latter is not required for the adjuvant action of poly IC

Another major pathway to Th1 immunity involves NK cells. NK cells are rapidly recruited to lymph nodes and splenic white pulp upon stimulation with mature DCs or some adjuvants like R-848 and poly IC, providing an early source of IFN- $\gamma$  required for Th1 polarization (Martin-Fontecha et al., 2004; Grégoire et al., 2008). In addition, the production of proinflammatory cytokines by NK cells has been associated with the adjuvant effect of poly IC (Salem et al., 2006). Specifically, engagement of NKp30 by DC induces secretion of proinflammatory cytokines by NK cells, e.g., TNF- $\alpha$ , which has been identified as an active inducer of DC maturation (Vitale et al., 2005). NK cells may have additional roles in inducing DC maturation, e.g., by direct cell contact and by selective killing of mature DCs that do not express optimal levels of MHC class I molecules (Ferlazzo et al., 2001). Several inflammatory cytokines stimulate NK cell proliferation and IFN- $\gamma$  production. For example, IL-12 synergizes with IL-18 or IL-15 to induce IFN- $\gamma$ , whereas IFN- $\alpha\beta$  promotes NK proliferation and cytotoxicity (Okamura et al., 1995; Yoshimoto et al., 1998; Fehniger et al., 1999; Kim et al., 2000; Chakir et al., 2001). In this paper, we have found that poly IC induces strong IFN- $\gamma$  production by NK cells and that this is fully dependent on the production of type I IFN *in vivo* by direct NK stimulation or through synergism with other cytokines, i.e., IL-18 (Matikainen et al., 2001). Yet IFN- $\gamma$  was not needed for CD4<sup>+</sup> Th1 responses or DC maturation, which was normal in mice genetically lacking IFN- $\gamma$  receptor. It remains possible that NK cells serve other roles in the adjuvant effects of poly IC, i.e., independent of type II IFN.



**Figure 8. Both hematopoietic and nonhematopoietic cells contribute to the adjuvant effect of poly IC.** (A) Gag p24-specific CD4<sup>+</sup> T cell responses were evaluated in bone marrow chimeras. WT and double TLR3.mda5<sup>-/-</sup> mice were lethally irradiated and injected with bone marrow cells from WT or deficient mice. After 6 wk, chimeras were primed and boosted 4 wk apart with 5  $\mu$ g  $\alpha$ -DEC-p24 and 50  $\mu$ g poly IC. After 1 wk, HIV gag-specific CD3<sup>+</sup>CD4<sup>+</sup> splenic T cells were analyzed for IFN- $\gamma$  secretion. Means  $\pm$  SD from two experiments are shown;  $n = 6$ . (B) Chimerism was assessed by expression of mda5 on CD11c<sup>+</sup> splenic cells. \*\*\*,  $P = 0.001$ .

### Innate type I IFNs in vivo induce DCs to stimulate adaptive immunity

The major challenge to understanding adjuvant function in vivo is, by definition, to understand the induction of adaptive immunity and how an early innate response to an adjuvant leads to adaptive immunity. Immunity induced by vaccination requires two broad functions at the level of DCs but needs to be studied in vivo. One is antigen presentation, which we enhanced by targeting the HIV gag protein to the DEC-205 antigen uptake receptor on a subset of DCs. Nevertheless, our mechanistic findings were similar when we administered higher doses of the antigen as an isolated gag p41 protein. The second function is that antigen-capturing DCs need to mature in vivo to induce immunity, as shown initially with another adjuvant,  $\alpha$ -galactosyl ceramide (Fujii et al., 2003). Additionally, maturation is required to avoid T cell tolerance upon targeting of antigens to DCs (Hawiger et al., 2001; Bonifaz et al., 2002; Kretschmer et al., 2006; Yamazaki et al., 2008). Our experiments show that type I IFN is important for DC maturation after poly IC. Poly IC and type I IFNs were each known to induce DC maturation, but their interdependence had not previously been tested in vivo. Likewise, Le Bon et al. (2001) found that type I IFNs could act on ex vivo-derived DCs, which then stimulates antibody immunity. We noted that poly IC not only up-regulates DC costimulatory molecules but allows DCs to become better inducers of MLR. The latter assay remains valuable to document that DCs have matured in the critical functional sense and not only by altering their cell surface phenotype. Both changes in DCs, i.e., up-regulation of CD86 and increased MLR stimulation, were abrogated by blockade of type I IFN receptors. Although ligation of PRRs has previously been associated with some features of DC maturation, we showed that this is not sufficient by itself and, additionally, the development of immunostimulatory function requires direct IFNAR signaling of the DCs.

### Both bone marrow-derived and nonhematopoietic cells need to respond to poly IC

Elegant studies have shown that antigen-presenting cells must themselves respond to TLR agonists to induce strong clonal expansion of antigen-specific TCR transgenic T cells (Spörri and Reis e Sousa, 2005; Nolte et al., 2007), but the response of naive mice and the contribution of radioresistant cells was not fully evaluated. Unexpectedly, we found that the adjuvant role of poly IC for a primary CD4<sup>+</sup> T cell response was severely reduced when either bone marrow-derived or radioresistant cells lacked the mda-5 and TLR3 PRRs. Radioresistant cells could include endothelial, epithelial, and stromal cells. The Langerhans type of DC is also radioresistant (Merad et al., 2002), but we found that depletion of these cells did not alter the levels of type I IFN detected in the serum. In contrast, we showed that stromal cells in vitro produced type I IFN as effectively as DCs upon poly IC stimulation, and in ongoing experiments, we find that poly IC markedly increases type I IFN mRNA in liver and lung cells suspensions that are depleted of CD11c<sup>+</sup> cells. These findings indicate

that adjuvants become more effective when they induce a systemic inflammatory response during the time that the antigen is captured and presented by DCs. In experiments that were not published, we found that the Th1 response was reduced if the administration of poly IC either preceded or followed the administration of protein antigen by 6 h. The systemic production of type I IFNs enhances two key innate responses in vivo: the maturation of DCs and the activation of NK cells, as well as key adaptive lymphocytes mediating delayed type hypersensitivity (Gallucci et al., 1999), humoral immunity (Le Bon et al., 2001), and CD8 T cell memory (Ferrantini et al., 1994). The further development of adjuvants may well need to consider the contributions of both hematopoietic and nonhematopoietic cells, which make innate cytokines that work directly on DCs so that they can act as initiators of antigen-specific adaptive immunity.

### MATERIALS AND METHODS

**Mice.** C57BL/6, BALB/c, and CxB6 F<sub>1</sub> were obtained from Harlan, 129/SvJ, IFN $\gamma$ R<sup>-/-</sup>, and IL-12 p40<sup>-/-</sup> were obtained from The Jackson Laboratory, STAT1<sup>-/-</sup> were provided by D. Levy (New York University, NY, NY), IFNAR<sup>-/-</sup> were provided by K. Murali-Krishna (University of Washington, Seattle, WA), and TLR3<sup>-/-</sup> were provided by S. Akira (Osaka University, Osaka, Japan). Mice were maintained under specific pathogen-free conditions and used at 7–8 wk of age according to Institutional Animal Care and Use guidelines. Animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC).

**In vivo cytokine secretion.** Mice were injected i.p. with 50  $\mu$ g poly IC (InvivoGen), 50  $\mu$ g Pam3cys (InvivoGen), 0.5  $\mu$ g Malp2 (Enzo Biochem, Inc.), 10  $\mu$ g LPS (*E. Coli*; Sigma-Aldrich), 50  $\mu$ g poly ICLC (Oncovir, Inc.), 50  $\mu$ g CpG ODN1826 (InvivoGen), or R-848 cream (2% topically applied to  $\sim$ 5 cm<sup>2</sup> of back skin; Celldex Therapeutics, Inc.), and serum was collected at the indicated times. Production of TNF- $\alpha$ , IL-12p40, IL-12p70, IL-6, IFN- $\gamma$  (eBioscience), and IFN- $\alpha$  and IFN- $\beta$  (R&D Systems) was determined by ELISA according to the manufacturer's instructions.

**Cell sorting and stromal cell isolation.** Spleens were cut in small pieces and incubated at 37°C for 30 min in Hank's medium supplemented with 400 U/ml collagenase D (Roche). EDTA (5 mM in final concentration) was added for the last 5 min. Cells were stained with biotinylated anti-CD3, anti-CD19, and anti-Ter119 mAbs (BD) for 20 min, washed, incubated with Streptavidin-coated magnetic beads, and passed through LD columns (Miltenyi Biotec). The negative fraction was collected and stained with Live/Dead Fixable Aqua viability dye (Invitrogen), FITC-anti-DX5, PE-anti-CD11c, PercPCy5.5-B220, Pacific Blue-anti-CD3, Alexa Fluor 750-anti-CD8 (eBioscience), and Alexa Fluor 647-DEC-205 (produced in house). Alternatively, cells were stained with Fixable Aqua viability dye, FITC-anti-Ly6G, PE-anti-CD115, PerCP-anti-F4/80, PE-Cy7-anti-CD11c, and Alexa Fluor 750-CD11b (eBioscience). Different populations were isolated by sorting on a FACS Aria with DiVa configuration (BD). Splenic stromal cells were obtained as previously described (Cheng et al., 2007). In brief, spleens were cut into small fragments, placed in a 48MW plate, and cultured in RPMI 1640 medium supplemented with 20% FCS and 1% MEM nonessential amino acids. After 1 mo, cells were further purified by CD45 negative selection.

**ELISA for antibody response.** To detect gag-specific antibody, high-binding ELISA plates (BD) were coated with 1  $\mu$ g/ml of gag protein overnight at 4°C, washed with PBS/0.1% Tween 20, and blocked with PBS/5% BSA for 1 h at 37°C. Serial dilutions of serum were added to the plates and incubated for another 1 h at 37°C. Plates were washed and incubated 1 h at 37°C with secondary HRP goat anti-mouse IgG antibody (Jackson Immuno-research Laboratories). TMB (3,3',5,5'-tetramethylbenzidine) substrate

solution (Thermo Fisher Scientific) was used as substrate. OD was measured in an ELISA reader at 450 nm.

**DC maturation and function.** Mice were injected i.p. with 100  $\mu$ g of anti-IFNAR blocking antibody or isotype control (Leinco Technologies) and 1 h later with PBS or 50  $\mu$ g poly IC. Spleens were collected 12 h later and collagenase digested. Maturation was monitored by increased expression of MHC-II (I-Ek), CD86, and CD40 after gating on CD11c<sup>hi</sup>DEC<sup>-</sup> or CD11c<sup>hi</sup>DEC<sup>+</sup> DCs. In mixed bone marrow chimera mice, expression of maturation markers was analyzed after gating on CD11c<sup>hi</sup>CD45.2<sup>+</sup> (IFNAR<sup>-/-</sup>) or CD11c<sup>hi</sup>CD45.2<sup>-</sup> DCs. To test DC stimulatory capacity, spleen CD11c<sup>+</sup> DCs were isolated 12 h after administration of poly IC with anti-IFNAR blocking antibody or isotype control. Alternatively, for mixed chimera mice, WT and IFNAR-deficient DCs were FACS sorted based on the expression of CD45.2. BALB/c DCs were fixed with 1% paraformaldehyde for 10 min on ice and graded numbers were added to  $2 \times 10^5$  CFSE-labeled (Invitrogen) C57BL/6 T cells. After 5 d of culture, samples were stained with Live/Dead Fixable Aqua viability dye and Pacific blue-anti-CD3 and acquired on an LSR II flow cytometer (BD).

**NK activation.** 2 h after 50  $\mu$ g poly IC i.p., spleen cell suspensions were prepared and incubated for 3 h with 10  $\mu$ g/ml brefeldin A (Sigma-Aldrich). Cells were stained with Live/Dead Fixable Aqua viability dye, FITC-anti-DX5, PE-anti-CD11c, and Pacific Blue-anti-CD3 mAbs. Cells were then fixed, permeabilized, and stained with APC-anti-IFN- $\gamma$  antibody. Sorted NK cells (CD3-DX5<sup>+</sup>) were cultured ( $3 \times 10^5$ ) with 50  $\mu$ g/ml poly IC or 2 ng/ml rIL-12 and 1 ng/ml rIL-15 (eBioscience).

**Bone marrow chimeras.** Recipient mice were  $\gamma$  irradiated twice with 500 rad, 3 h apart. 3 h later, they were reconstituted with  $3 \times 10^6$  marrow cells that had been harvested from the femurs and tibias of age-matched mice. After 6 wk, mice were tested for chimerism by RT-PCR for mda5 on CD11c<sup>+</sup> cells (InvivoGen) or by flow cytometry, based on the expression of CD45.1 and CD45.2 (>98%).

**Assays for HIV-specific CD4<sup>+</sup> T cells.** Mice were immunized twice i.p. at 4-wk intervals with 5  $\mu$ g HIV gag-p24 and 10  $\mu$ g HIV gag-p41 (Trumpfheller et al., 2008) together with adjuvant, which was 50  $\mu$ g poly IC, 50  $\mu$ g Pam3cys, 0.5  $\mu$ g Malp-2, 10  $\mu$ g LPS, 50  $\mu$ g poly ICLC, and R-848 cream (topic, 5 cm<sup>2</sup>) or 50  $\mu$ g CpG ODN1826. For IFNAR1 blocking, mice were simultaneously injected days 0 and 2, with 100  $\mu$ g of anti-IFNAR blocking antibody or isotype control. 1 wk later, splenocytes were restimulated with 2  $\mu$ M p24 or negative control peptide mix, along with 2  $\mu$ g/ml of costimulatory  $\alpha$ -CD28 (clone 37.51) for 6 h. 10  $\mu$ g/ml brefeldin A was added for the last 5 h. Cells were washed, incubated 10 min at 4°C with 2.4G2 mAb to block Fc $\gamma$  receptors, washed, and stained with Live/Dead Fixable Aqua viability dye, Pacific blue-conjugated anti-CD3, PerCP-conjugated anti-CD4, and Alexa Fluor 750-conjugated anti-CD8 mAbs for 20 min at 4°C. Cells were permeabilized (Cytofix/Cytoperm Plus; BD) and stained with APC-anti-IFN- $\gamma$ , PE-anti-IL-2, and PE-Cy7 anti-TNF- $\alpha$  mAbs for 15 min at room temperature (BD). Samples were acquired on an LSR II flow cytometer and analyzed with FlowJo Software (Tree Star, Inc.).

**Statistical analysis.** Statistical significance was evaluated using a two-tailed Student's *t* test with a 95% confidence interval. Results are expressed as means  $\pm$  SD. In the figures, *p*-values of 0.001 are labeled with a triple asterisk. Analysis was performed with a Prism 3 program (GraphPad Software, Inc.).

**Online supplemental material.** Fig. S1 shows the frequency of IL-2 and TNF- $\alpha$ -producing CD4<sup>+</sup> T cells after DEC-p24 immunization with different adjuvants. Fig. S2 demonstrates the mda-5 dependence of the production of type I IFN in serum. Fig. S3 shows the gating strategy for the MLR assays. Fig. S4 shows representative dot plots from Fig. 7.

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