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Influenza Vaccines Differentially Regulate the Interferon Response in Human Dendritic Cell Subsets

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Abstract

Human dendritic cells (DCs) play a fundamental role in the initiation of long-term adaptive immunity during vaccination against influenza. Understanding the early response of human DCs to vaccine exposure is thus essential to determine the nature and magnitude of maturation signals that have been shown to strongly correlate with vaccine effectiveness. In 2009, the H1N1 influenza epidemics fostered the commercialization of the non-adjuvanted monovalent H1N1 California vaccine (MIV-09) to complement the existing non-adjuvanted trivalent Fluzone[®]09–10 vaccine (TIV-09). In retrospective studies, MIV-09 displayed lower effectiveness than TIV-09. Here, we show that TIV-09 induces monocyte-derived DCs (moDCs), blood conventional DCs (cDCs) and plasmacytoid DCs (pDCs) to express CD80, CD83, and CD86, and secrete cytokines. TIV-09 stimulated the secretion of type I interferons (IFN) IFN- α and IFN- β and type III IFN IL-29 by moDCs and cDCs subsets. The vaccine also induced the production of IL-6, TNF and the chemokines IP-10 and MIP-1 β . Conversely, MIV-09 did not induce the production of type I IFN in moDCs and blood cDCs. Furthermore, it inhibited the TIV-09-induced secretion of type I IFN by these DCs. Yet, both vaccines induced pDCs to secrete type I IFN indicating that different influenza vaccines activate distinct molecular signaling pathways in DC subsets. These results suggest that subtypes of non-adjuvanted influenza vaccines trigger immunity through different mechanisms and that the ability of a vaccine to induce an IFN response in DCs may offset the absence of adjuvant and increase vaccine efficacy.

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For all experiments where $n < 20$, please include the source/primary data (see Checklist) as a separate Excel file; this is easily done with the data organized by Figures on separate tabs

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INTRODUCTION

Vaccination is the most efficient way to protect humans against influenza. Several influenza vaccines are currently available including split subunit vaccines and attenuated viral vaccines (1). Split vaccines, which are most commonly used, are prepared by culture of the influenza virus in hen eggs or cell cultures, followed by purification and treatment with detergents (2). In the US, split vaccines are used without adjuvants while in Europe, some vaccines contain adjuvants such as MF59 or AS03 (3, 4). Because the composition of influenza vaccines varies annually based on results from global influenza surveillance data, the determination of vaccine effectiveness remains a continuous challenge.

The need for rapid implementation of a vaccination program during the outbreak of the pandemic influenza A(H1N1)pdm09 virus in 2009 led initial studies to focus on immunogenicity rather than overall efficacy of the vaccine. Several influenza vaccines were then marketed, including the trivalent non-adjuvanted seasonal flu vaccine Fluzone®09–10 (hereafter TIV-09) containing A/Brisbane/59/2007 (H1N1), A/Brisbane/10/2007 (H3N2)-like and B/Brisbane/60/2008 viral strains and the monovalent H1N1 vaccine (hereafter MIV-09) containing A/California/7/2009 (H1N1)-like virus. The monovalent vaccine licensed in Europe and Canada was adjuvanted while the one licensed in Australia and the US was not. Subsequent vaccine trials performed with TIV-09 or the adjuvanted forms of MIV-09 demonstrated efficacy (5–9). In contrast, the non-adjuvanted form of MIV-09 displayed lower effectiveness, low seroprotection in healthy children (35%) and suboptimal response in solid organ transplant (SOT) recipients and children with systemic lupus erythematosus (SLE) (9–13). It is unclear why non-adjuvanted TIV-09 and MIV-09, two vaccines produced by the same manufacturer (Sanofi-Pasteur), display such disparate efficacy levels.

Traditionally, vaccine efficacy has been measured by hemagglutinin inhibiting antibody response as a correlate of humoral immunity (14). Some studies suggested that assessment of T-cell responses might provide a better correlate of vaccine protection against influenza, especially in the elderly (15). Other studies suggested that influenza vaccines might differentially activate subpopulations of immune cells (16, 17). We and others demonstrated that administration of seasonal influenza vaccines to healthy volunteers induces global transcriptional changes in whole blood that can be followed at discrete time points (18–23). In particular, a transcriptional signature of interferon (IFN)-inducible genes was observed between 12 and 36h post-vaccination, followed by a plasmablast signature at day 7 (23). This early signature may represent an important correlate of vaccine efficacy, as type I IFNs enhance antigen cross-presentation to CD8+ T cells, foster T-helper 1 differentiation, support B cell differentiation into antibody-producing plasmablasts and induce dendritic cell (DC) maturation (24–26). DCs play an essential role in vaccination, by detecting and presenting foreign antigens to adaptive immune cells (27, 28). We recently showed that different DC subsets, including monocyte-derived DCs (moDCs) and blood-derived conventional DCs (cDCs), respond differently to various bacterial and viral vaccines (29).

To better understand the biological mechanisms underlying the difference in effectiveness of non-adjuvanted TIV-09 and MIV-09, we compared their ability to activate human DC

subsets. Our results suggest that in the absence of adjuvant, TIV-09 activates a broader range of DC subsets than MIV-09 in a type I IFN-dependent manner, possibly explaining the increased efficacy of the former vaccine.

RESULTS

TIV-09 but not MIV-09 activates moDCs and induces type I IFN secretion

To assess the effects of TIV-09 and MIV-09 on DC activation, moDCs were obtained by culturing isolated human monocytes in the presence of GM-CSF and IL-4 for 6 days, and subsequently exposed to each vaccine for 18h. TIV-09 upregulated the surface expression of hallmark DC maturation markers CD40, CD80, CD83, CD86, HLA-DR, PD-L1, PD-L2 and CCR7 (Fig. 1A, n=7). Compared to TIV-09, MIV-09 induced significantly ($p<0.01$) lower expression of these markers. moDC activation was further assessed by measuring the release of cytokines at different time points following vaccine challenge. IFN- β reached its peak level at 12h and decreased to near baseline values after 48h, suggesting its consumption. MIP-1 β reached a peak at 12h and slightly decreased thereafter. Other cytokines such as IFN- α , IL-29, IL-6 and IP-10 attained peak levels by 12–24h and remained stable at 48h. Conversely, MIV-09 did not induce cytokine secretion, with the exception of MIP-1 β (Fig. 1B, n=10). Because TIV (45 μ g) contains three times more viral protein than MIV-09 (15 μ g), a dose-dependent analysis was conducted. Even at high concentration (>4-fold), MIV-09 failed to induce the secretion of IFN- β and IP-10 by moDCs, suggesting that protein concentration cannot explain the observed differences (Fig. S1). Staining with propidium iodide and Annexin V at 4, 8, 12 and 24h also revealed that the lack of cytokines observed with H1N1 was not due to vaccine-induced cell death (Fig. 1C, n=6). We further assessed IP-10 production by moDCs in the presence of other inactivated viral vaccines such as Human Papilloma Virus (Merk), Hepatitis A, Hepatitis B (GSK) and Rabies (Sanofi Pasteur) (Table S1). Only TIV-09 induced secretion of IP-10 by moDCs (Fig. S2).

Gene expression profiling was performed by microarray on moDCs from six healthy individuals cultured for 6h with TIV-09 or MIV-09. TIV-09 upregulated all known IFN- α transcripts, IFN- β , IFN- ω and IL-29 (Fig. 1D, n=6). Consistent with the protein data, MIV-09 failed to increase the transcription of type I and III IFN genes. In addition, TIV-09 upregulated the expression of other pro-inflammatory and regulatory cytokines including TNF, IL-6, IL-1 β and IL-10, which were also slightly upregulated in response to MIV-09. Taken together, these results indicate that the seasonal Influenza vaccine TIV-09, but not MIV-09, potently activates moDCs including induction of type I and III IFNs.

Cell-intrinsic type I IFN production contributes to moDC activation

Upon activation, several cell types first transcribe the IFN- β gene, which results in the prompt secretion of IFN- β . IFN- β then binds to the IFN receptor (IFNAR), consequently initiating IFN- α production (30, 31). To determine whether the early secretion of IFN- β by moDCs might contribute to their further activation, moDCs were exposed to TIV-09 in the presence of a blocking anti-IFN- α/β 2 monoclonal antibody. Activation was assayed by measuring the secretion of the IFN-inducible cytokine IP-10. Addition of anti-IFN- α/β 2 antibody abolished IP-10 production and strongly reduced production of IFN- α and IL-29

without affecting that of IFN- β (Fig. 2A, n=4). In addition, IFNAR blockade resulted in the upregulation of IL-1 β , TNF, and IL-10, suggesting an autocrine inhibitory loop (32). The addition of anti-IFN- α / β R2 antibody also partially inhibited the upregulation of CD80, CD83, CD86, CD70, and CD40 (Fig. 2B, n=4), suggesting that moDC maturation is dependent on type I IFN secretion. These data indicate that the early burst of IFN- β production contributes to TIV-09-induced activation of moDCs.

MIV-09 inhibits the IFN response in moDC

We next analyzed the effects of adding the H1N1 strain A/California/07/2009 X-179A in vaccines by testing several Fluzone® batches prepared over different years on moDCs. Fluzone® vaccines from years 2006–2007, 2008–2009, and 2009–2010, composed of three closely related influenza strains (see Methods) induced moDCs to secrete similar levels of IP-10 (Fig. 3A, n=7). However, Fluzone® 2010–2011, which contains the H1N1 strain A/California/07/2009 X-179A, failed to induce IP-10 secretion by moDCs.

To determine whether MIV-09 could inhibit moDC activation, we activated moDCs with TIV-09 or selected TLR agonists, including LPS (TLR4 ligand), R848 (TLR7/8 ligand), CL097 (TLR7/8 ligand) and Poly(I:C) (TLR3 and RIG-I/MDA5 ligand), with or without MIV-09. At a concentration of 25 μ g/mL, MIV-09 not only completely abrogated the secretion of IP-10 induced by TIV-09, but also the secretion of other IFN-related cytokines including IFN- β , IL-29 and IL-6 (Fig. 3B, 3C, n=7). MIV-09 also inhibited the secretion of cytokines by moDCs activated by CD40L and all TLR agonists tested. As MIV-09 could inhibit the early IFN- β response, we wondered whether addition of exogenous recombinant IFN- α might reverse the inhibitory effects of MIV-09 on the secretion of other cytokines, which was not the case (Fig. 3C, n=7). MIV-09 also prevented the expression of CD80, CD83, CD86, CD70 and CD40 induced by TIV-09 and all tested DC-activating stimuli (Fig. 3D, n=4). These results indicate that MIV-09 acts as a potent inhibitor of cytokine secretion and maturation in activated moDC.

To further understand the molecular alterations induced by both vaccines at the pathway level, we conducted Ingenuity Pathway Analysis (IPA) on the transcriptional fingerprints of vaccine treated-cells (Fig 3E). The top pathways associated with TIV-09 exposure included IFN signaling (yellow, blue), protein ubiquitination (blue), interferon regulatory factor (IRF) signaling (yellow) and retinoic acid-mediated signaling (yellow). These pathways were suppressed when moDCs were exposed to MIV-09. On the other hand, MIV-09 enhanced the ERK/MAPK signaling pathway (purple), which can inhibit IFN responses (33). These results suggest that MIV-09 inhibits IFN signals and moDC maturation by skewing the transcriptional cascade of vaccine-exposed moDC towards ERK-mediated pro-inflammation.

TIV-09 elicits a type I IFN signature in blood cDCs

As moDCs and blood cDCs differ phenotypically and functionally, we next assessed the effects of TIV-09 and MIV-09 on sorted *ex vivo* blood cDCs. As observed in moDCs, the surface expression of CD80, CD83, CD86 and HLA-DR was increased on cDCs exposed for 18h to TIV-09. Exposure to MIV-09 was associated with lower induction of CD80, CD83 and CD86 (Fig. 4A, n=4). While cDCs express significantly lower levels of type I IFN than

pDC (34), cDCs exposed to TIV-09, but not to MIV-09, displayed upregulation of both IFN-inducible transcripts and IP-10 secretion (Fig. 4B, n=3 and 4C, n=6). As observed with moDCs, MIV-09 prevented the TIV-09-dependent secretion of IP-10 by cDCs (Fig. 4D, n=6) and downregulated TIV-09-induced surface expression of CD83, CD86 and HLA-DR (Fig. 4E, n=4). These data suggest that MIV-09 also blocks IFN signals in *ex vivo* cDCs.

Both vaccines elicit a type I IFN signature in blood pDCs

Plasmacytoid DCs (pDCs) promptly secrete large amounts of type I IFN in response to a number of stimuli including viruses (35). Therefore, we exposed pDCs to TIV-09 or MIV-09 for 18h and assessed the expression of activation markers by FACS and IFN transcription by microarray. Both vaccines upregulated CD80, CD86 and HLA-DR expression with TIV-09 being consistently more potent than MIV-09 (Fig. 5A, n=4). Transcriptional analysis revealed that both vaccines upregulated type I IFN transcripts (Fig. 5B, n=3). MIV-09 was less efficient than TIV-09 at inducing the secretion of IP-10 and IFN- β (Fig. 5C, n=6, 5D, n=6). When the two vaccines were combined, MIV-09 suppressed TIV-09-induced IP-10 production by about 70% (Fig. 5E, n=6) and downregulated TIV-09-induced surface expression of CD83 (Fig. 5F, n=4).

MIV-09 fails to induce a type I IFN signature *in vivo*

To better understand the early immune response to influenza vaccination *in vivo*, we vaccinated healthy individuals with TIV-09, MIV-09 or saline control and assessed the transcriptional profiles of whole blood at 0h, 1.5h, 3h, 6h, 9h, 12h, 15h, 24h, 36h, 2d, 3d and 7d post-vaccination by microarray. Data were analyzed both at the single transcript level and using a previously described framework of modules of co-expressed transcripts, focusing on three IFN modules (36). Consistent with previous studies from our group (18) as well as others (19–23), TIV-09 induced a transient type I IFN signature 24–48h post vaccination. This signature was however not induced in response to MIV-09 (Fig. 6). These results indicate that MIV-09 fails to induce an IFN response *in vivo*.

DISCUSSION

This study focuses on how non-adjuvanted influenza vaccines that display different effectiveness affect the biological functions of human DC subsets. We first show that the Fluzone® vaccines that do not contain the [A/California/07/2009 X-179A (H1N1)] H1N1 Influenza strain activate moDCs and blood cDCs as revealed by secretion of type I IFN, IL-6, IL-29 and MIP-1b and increased surface expression of several DC activation markers. Blocking IFN signaling with an anti-IFNAR antibody further revealed the partial dependence of this activation on an early wave of IFN- β production by DCs. TIV-09 also induced pDCs to transcribe and secrete type I IFNs. We then show that the monovalent non-adjuvanted MIV-09, which displayed limited clinical efficacy, i) did not induce an IFN response and maturation of moDCs and cDCs; ii) blocked the IFN-mediated activating effects of TIV-09 *in vitro*, and IFN signaling *in vivo*. These results suggest that in the absence of adjuvant, certain vaccines but not others retain immunogenic properties through IFN-mediated DC activation.

These findings might partly explain why *in vivo* administration of TIV-09 results in the short-term expression of an IFN signature in circulating blood cells. Type I IFNs play a pivotal role in the induction of adaptive immune responses by promoting the expansion of CD4⁺ and CD8⁺ T cells and the maturation of B cells into plasmablasts (24, 26). High vaccine responses correlate with increased early expression of interferon signaling and antigen processing and presentation (21). Furthermore, type I IFNs act as powerful adjuvants when administered with influenza vaccine to mice (37). Thus, one could speculate that the antibody response elicited against TIV-09 might at least in part be due to this early IFN response.

MIV-09, on the other hand, was not able to induce moDC and blood cDC activation as measured by secretion of cytokines including type I IFN and induction of activation markers. Moreover, MIV-09 acted as a powerful inhibitor of the effects of TIV-09 on the production of cytokines, including IFN and induction of activation markers by moDCs and cDCs. Instead, exposure to MIV-09 was associated with a unique transcriptional signature in moDCs that was enriched for ERK/MAPK signaling. Studies with murine macrophages indicate that IFN- β production is inhibited through ERK activation (33), thereby providing a plausible mechanism for the IFN-inhibitory activity of MIV-09 in moDCs and blood cDC. The inability of MIV-09 to completely shut down the IFN machinery in pDCs may be due to constitutive expression of IRF7 in these cells (38). Moreover, the combination of MIV-09 with TIV-09 results in production levels of IP-10 that are lower than those induced by TIV-09. These results suggest that MIV-09 has the potential to activate the IFN transcription cascade but this action is blocked by the presence of an inhibitory element in this vaccine.

Comparative studies between TIV-09 and the 23-valent pneumococcal vaccine Pneumovax®23 demonstrated that the two vaccines induce protective antibody responses but differ in the blood transcriptional profiles they elicit (18). When assessing IP-10 production by moDCs in the presence of other inactivated viral vaccines including Human Papilloma Virus, Hepatitis A, Hepatitis B and Rabies (Table S1), only TIV-09 induced secretion of IP-10 by moDCs (Fig. S2). Most seasonal influenza vaccines have been shown to exhibit early IFN signatures (18, 21–23), suggesting that type I IFN plays an important role in shaping protective immunity against influenza. Our study shows that unlike seasonal influenza vaccines, MIV-09 fails to exhibit the classic type I signature *in vitro* as well as *in vivo*.

Higher effectiveness has been observed in MIV-09 supplemented with squalene-based adjuvants MF59 and AS03 as compared with its non-adjuvanted form (3, 4). Several observations support a direct effect of MF59 on DCs. MF59 rapidly localizes to intracellular DEC-205⁺ DC compartments after injection in mice (39), promotes recruitment of human myeloid cells to the injection site, enhances DC maturation and antigen uptake and facilitates DC migration to draining lymph nodes (40). A recent study in mice also demonstrated that MF59 promotes the retention of unprocessed antigen within the lymph nodes, thereby facilitating antigen encounters with follicular DCs (41). Whether these two adjuvants can induce the IFN response in DCs is still unknown.

Due to the presence of broadly cross-reactive antibody responses as a result of prior vaccination or infection (42, 43), it will be challenging to determine whether the lack of *in vitro* and *in vivo* activation of type I IFN signature reflects the reduced immunogenicity of MIV-09 as compared to TIV-09. Vaccines against *Pneumococcus* or Rabies are fully immunogenic *in vivo* despite lacking interferogenic properties in DC *in vitro* (29). These observations suggest the existence of IFN-dependent and IFN-independent mechanisms for mounting immune responses against specific pathogens.

Our study is limited by its *in vitro* nature. Our model of vaccine-challenged monocyte-derived and sorted blood DC does not fully represent molecular and cellular events occurring *in situ* after vaccination. In particular, it does not account for DC activation mechanisms that can be triggered indirectly through the recruitment of other inflammatory cell populations. The phenotype of the DC populations studied may also not accurately reflect the phenotype of DCs found in skin and muscle, especially that of Langerhans cells. Finally, these studies are complicated by the evolving nature of the influenza vaccine and the scarcity of vaccines from earlier years.

Altogether, this study sheds light on the mechanisms of DC activation by influenza vaccines. Furthermore, it highlights the differences between non-adjuvanted influenza vaccines in inducing IFN and pro-inflammatory responses *in vitro* and *in vivo*, further unraveling the mechanisms of action of vaccines at the systemic level and the role of early inflammatory responses in the process of vaccination.

METHODS

Subjects and study design

This study was approved by the Baylor Institute for Immunology Research Institutional Review Board (BIIR IRB 009-282). Written informed consent was obtained for all subjects. For *in vitro* studies, cells were obtained from apheresis of 10 healthy individuals (6 females, 4 males) aged 27 to 59 years. For the vaccination study, healthy adults, aged 18 to 64 years, were enrolled to receive a single intra-muscular dose of TIV-09 [A/Brisbane/59/2007 IVR 148 (H1N1), A/Uruguay/716/2007 NYMC X-175C (H3N2), B/Brisbane/60/2008], MIV-09 [A/California/07/2009 X-179A (H1N1)] or placebo (saline) (n=3 per group). Exclusion criteria included pregnancy, active allergy, or vaccinations within the previous two months. Blood was drawn by finger prick at 0h, 1.5h, 3h, 6h, 9h, 12h, 15h, 24h, 36h, 2d, 3d and 7d. Power analysis was conducted to estimate sample size for moDC experiments based on preliminary results from microarray, luminex and FACS analyses. With $N=6$, the study was adequately powered (>0.8) to detect a 3-fold difference when as few as 2000 genes are differentially expressed (microarray), a 2-fold difference in secreted proteins (Luminex) and a change 7.5% of positive cells for the surface markers considered (FACS).

In vitro DC culture and stimulation

Monocyte-derived DCs (moDCs) were generated from monocytes isolated from healthy donor apheresis fractionated by elutriation. Monocytes were enriched by negative selection using EasySep Human Monocyte Enrichment without CD16 Depletion Kit (Stemcell

Technologies) and cultured in CellGro DC media (CellGenix) with 1% penicillin/streptomycin. Cells were cultured in sterile 50 mL tissue culture bags (AFC) at an initial density of 1×10^6 cells/mL along with GM-CSF at 10 ng/mL and IL-4 (R&D systems) at 50 ng/mL for 6 days. Cells were supplemented with additional cytokine doses on days 2 and 4. On day 6, DCs were harvested and further cultured in 200 μ L of RPMI 1640 supplemented with (1% glutamine, 1% penicillin/streptomycin, 1% HEPES, 1% non-amino-acids, 1% sodium pyruvate and 0.1% β -mercaptoethanol) (Invitrogen) and 10% fetal bovine serum (FBS) at a density of 1×10^5 cells/mL in 96-well plates. TIV-09 (45 μ g viral protein, 6 μ L/mL) and/or MIV-09 (15 μ g viral protein, 6 μ L/mL) (Sanofi Pasteur) was added, and cells were incubated at 37°C for 0, 6, 12, 24 and 48h. Supernatants were collected and stored at -80°C.

Another set of experiments was performed with Fluzone® from different vaccination seasons. moDCs were exposed for 8h to one of the following vaccines, all from Sanofi Pasteur: i) Fluzone®2006–2007 [A/New Caledonia/20/99 (H1N1), A/Wisconsin/67/05 (H3N2), B/Malaysia/250604], ii) Fluzone®2008–2009 [A/Brisbane/59/2007 (H1N1), A/Brisbane/10/2007 (H3N2), B/Florida/04/2006], or iii) Fluzone®2010–2011 ([A/California/07/2009 X-179A (H1N1), A/Victoria/210/2009 X-187 (H3N2), B/Brisbane/60/2008]. Supernatants were collected and stored at -80°C.

Separate experiment was performed to assess IP-10 production by moDCs in the presence of other inactivated viral vaccines such as Human Papilloma Virus (Merk), Hepatitis A, Hepatitis B (GSK) and Rabies (Sanofi Pasteur). moDCs were treated with vaccines for 18h. Supernatants were stored at -80°C.

DC phenotype and cytokine assay

DCs (1×10^5) were stimulated with selected vaccines for 18h, and labeled with fluorochrome-conjugated anti-CD83, anti-CD80, anti-CD86, anti-HLA DR, anti-PD-L1, anti-PD-L2, anti-CCR7, anti-CXCR4 and anti-CD40 antibodies (BD Biosciences). Cell death was assessed by Annexin V and PI staining (BD Biosciences). Labeled cells were analyzed using a FACS Canto (BD Biosciences). IP-10 (BD Biosciences) and IFN- β (R&D Systems) levels were measured by ELISA, and other cytokines were assessed using bead-based cytokine multiplex analysis (Luminex, Bio-Rad).

Purification and activation of pDCs and mDCs

Buffy coats obtained from healthy donors (Carters Blood Care) were fractionated on a Ficoll gradient. Peripheral blood mononuclear cells (PBMCs) were further enriched for DCs through negative selection using the Pan DC kit (Stemcell Technologies). The enriched cells were sorted on a FACS Aria (BD Biosciences) for LIN⁻/HLA-DR⁺/CD123⁺ and CD11c⁻ pDCs and LIN⁻/HLA DR⁺/CD123⁻ and CD11c⁺ mDCs (Fig. S3). Purity was routinely >98%. DCs were stimulated at a density of 5×10^4 cells per well with vaccines (6 μ L/mL) in 200 μ L complete medium in 96-well plates for 0, 6, 16, 24 and 48h. For microarray analysis, 0.5×10^6 cells were cultured with (6 μ L/mL) vaccine in 0.5 mL complete medium for 6h, and processed for RNA analysis.

RNA processing and transcriptional analysis

After vaccine stimulation, cells were lysed in RLT buffer (RNeasy Plus Mini Kit, Qiagen), 1% β -mercaptoethanol and stored at -80°C . Total RNA was isolated from cell lysates according to the manufacturer's instructions. RNA from samples passing quality control was amplified and labeled with Illumina TotalPrep RNA amplification kit (Illumina). RNA integrity was assessed using an Agilent 2100 Analyzer (Agilent Technologies). Biotinylated complementary RNA (cRNA) was hybridized to Illumina Human-6 Beadchip Array version 2 and scanned in Illumina Beadstation 500. Fluorescent hybridization signals were assessed with Beadstudio software (Illumina). Statistical analysis and hierarchical clustering were performed using GeneSpring 7.3.1 software (Agilent Technologies). For the vaccinated healthy cohort, data were normalized to the median of the $t=0\text{h}$ samples across donors.

Statistical analysis

Data from cell culture experiments were expressed as mean of replicate experiments. Statistical significance was determined by non-parametric test such as Mann Whitney test. $p<0.05$ was considered statistically significant. For microarray analysis, unless otherwise specified, we conducted Welch's unequal variances t-test ($p<0.01$) adjusting the FDR with the Benjamini-Hochberg procedure. For all figures, horizontal lines or bars represent the mean, and whiskers the standard deviation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. TIV-09, but not MIV-09, activates moDCs

(A) moDCs were stimulated with TIV-09 (6 μ L/mL), MIV-09 (6 μ L/mL) or media for 18h. Surface expression of moDC activation markers was assessed by flow cytometry ($N=7$). (B) moDCs were cultured in the presence of TIV-09 or MIV-09 (6 μ L/mL) for 0, 6, 12, 24 and 48h. Supernatants were harvested for quantification of cytokines by Luminex ($N=10$). Statistics shown represent the comparison between TIV-09 and MIV-09 at each time point. (C) moDCs were stained with Annexin V and PI to examine cell death at 4h, 8h, 12h and 24h post-vaccine treatment ($N=6$). (D) Transcriptional analysis of IFNs and other cytokines

in moDCs cultured with TIV-09 or MIV-09 for 6h. Data were normalized to media control ($N=6$). (Mann-Whitney test; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

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Figure 2. TIV-09 is a strong inducer of type I IFN

(A) moDCs were treated with increasing doses of TIV-09 with or without anti-IFNAR antibody (10 μ g/mL) for 8h. Supernatants were assessed by Luminex for the presence of type I IFN and inflammatory cytokines. Statistics shown represent the comparison between TIV-09 and TIV-09 with IFN α / β R2 antibody at each concentration. (Mann-Whitney test; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$). (B) moDCs were stimulated with TIV-09 (6 μ L/mL) with or without anti-IFNAR antibody for 18h. Surface expression of DC markers was assessed by flow cytometry. The figure is representative of experiments in four independent donors.

Figure 3. MIV-09 inhibits type I IFN

(A) moDCs were treated with increasing doses of Influenza vaccines used during the 2006–2007, 2008–2009, 2009–2010 and 2010–2011 seasons. The graph summarizes data from seven donors. (B) moDCs were treated with 25 $\mu\text{L/mL}$ TIV-09 combined with increasing doses of MIV-09 for 8h. Supernatants were harvested for ELISA. The graph summarizes data from seven donors. (C) moDCs were activated with TIV-09 (6 $\mu\text{L/mL}$), LPS (50 ng/mL), CD40L (1 $\mu\text{g/mL}$), R848 (3 $\mu\text{g/mL}$), or CL097 (5 $\mu\text{g/mL}$) alone or with MIV-09 (10 $\mu\text{L/mL}$) with or without recombinant IFN- α (2000 U/mL). Supernatant cytokine

concentrations were assessed by Luminex. The graph summarizes data from seven donors. **(D)** moDCs were stimulated with TIV-09, LPS, CD40L, R848 or CL097 with or without MIV-09. The surface expression of moDC activation markers was examined by flow cytometry. The figure is representative of experiments in four independent donors. **(E)** Ingenuity Pathway Analysis of the transcripts unique to each vaccine and most highly expressed after vaccine exposure. The top three canonical pathways enriched in the transcripts induced by each vaccine are shown ($N=3$). (Mann-Whitney test; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

Figure 4. TIV-09 activates cDCs

(A) Sorted blood cDCs were stimulated *in vitro* for 18h with media alone, TIV-09 or MIV-09. Surface expression of activation markers was examined by flow cytometry. The figure is representative of experiments in four independent donors. (B) Transcriptional analysis of IFNs in cDCs stimulated with TIV-09 or MIV-09 for 6h. Data were normalized to medium control ($N=3$) (C) cDCs were activated *in vitro* with media, TIV-09 or MIV-09 for 0, 6, 24 and 48h. IP-10 levels were measured by ELISA. The graph summarizes data from six donors (D) cDCs were treated with TIV-09 (6 μ L/mL), combined with different doses of MIV-09 for 8h. Supernatants were harvested and IP-10 was measured by ELISA. The graph summarizes data from six donors (E) cDCs were stimulated with TIV-09 in the presence or absence of MIV. Surface expression of CD80, CD86, CD83 and HLA-DR was measured by flow cytometry. The figure is representative of a result from experiments on four independent donors. (Mann-Whitney test; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$).

Figure 5. TIV-09 activates pDCs

(A) Sorted pDCs were activated *in vitro* with TIV-09 or MIV-09 or control media for 18h and surface expression of activation markers was measured by flow cytometry. The figure is representative of experiments in four independent donors (B) Transcriptional analysis of IFNs in pDCs stimulated with TIV-09 or MIV-09 for 6h. Data were normalized to medium control ($N=3$) (C, D) pDCs were activated *in vitro* with media, TIV-09 or MIV-09 for 0, 6, 24 and 48h. IP-10 and IFN- β levels were measured by ELISA. (E) pDCs were treated with TIV-09 (6 μ L/mL), combined with different doses of MIV-09 for 8h. Supernatants were harvested and IP-10 levels were measured by ELISA. Figs 5 C, D and E summarize data from six donors. (F) pDC were stimulated with TIV-09 in the presence or absence of MIV surface expression of surface expression of CD80, CD86, CD83 and HLA-DR, measured by

flow cytometry. The figure is representative of a result from experiments on four independent donors. (Mann-Whitney test; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

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Figure 6. TIV-09 but not MIV-09 induces an IFN signature in the blood of vaccinated individuals at day 1

Healthy individuals were vaccinated at $t=0h$ with saline, TIV-09 or MIV-09 ($N=3$ per group), and blood was drawn at 0h, 1.5h, 3h, 6h, 9h, 12h, 15h, 24h, 36h, 2d, 3d and 7d. Data were normalized to the median of the 0h samples across donors. Transcripts from three IFN blood modules (M1.2, M3.4 and M5.12) (36) were selected for analysis. Both transcript-level and module-level analyses are displayed. Module expression is calculated as the percentage of transcripts from a specific module that are over or underexpressed in a specific condition as compared to the 0h reference control.