Influenza Virus Vaccination Induces Interleukin-12/23 Receptor β1 (IL-12/23Rβ1)-Independent Production of Gamma Interferon (IFN-γ) and Humoral Immunity in Patients with Genetic Deficiencies in IL-12/23Rβ1 or IFN-γ Receptor 1

Tjitske de Boer,¹ Jaap T. van Dissel,¹ Taco W. J. Kuijpers,² Guus F. Rimmelzwaan,³ Frank P. Kroon,¹§ and Tom H. M. Ottenhoff§* ¹

Department of Infectious Diseases, LUMC, Leiden, The Netherlands; ²Emma Children’s Hospital at the Academic Medical Center (AMC), Amsterdam, The Netherlands; and ³Department of Virology, EUR, Rotterdam, The Netherlands

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To investigate whether protective immune responses can be induced in the absence of normal interleukin-12/23/gamma interferon (IL-12/23/IFN-γ) axis signaling, we vaccinated with the seasonal influenza virus subunit vaccine two patients with complete IL-12/23 receptor β1 (IL-12Rβ1) deficiencies, two patients with partial IFN-γ receptor 1 (pIFN-γRI) deficiencies, and five healthy controls. Blood samples were analyzed before, 7 days after, and 28 days after vaccination. In most cases, antibody titers reached protective levels. Moreover, although T-cell responses in patients were lower than those observed in controls, significant influenza virus-specific T-cell proliferation, IFN-γ production, and numbers of IFN-γ-producing cells were found in all patients 7 days after the vaccination. Interestingly, influenza virus-specific IFN-γ responses were IL-12/23 independent, in striking contrast to mycobacterium-induced IFN-γ production. In conclusion, influenza virus vaccination induces IL-12/23-independent IFN-γ production by T cells and can result in sufficient humoral protection in both IL-12Rβ1- and pIFN-γRI-deficient individuals.

Gamma interferon (IFN-γ) is a key cytokine in the cell-mediated immune response and is produced by type 1 T cells and NK cells. Its production is induced mainly by interleukin-12 (IL-12), a heterodimeric cytokine that consists of a p40 and a p35 chain, which binds to IL-12 receptor β1/β2 (IL-12Rβ1/β2) receptor complexes at the cell surfaces of T cells and NK cells. The related heterodimeric cytokine IL-23 contains the same p40 subunit as IL-12, but coupled to a unique p19 subunit, and binds to a receptor consisting of the IL-12Rβ1 chain complexed to the IL-23R protein. Both IL-12 and IL-23 are produced by activated macrophages and dendritic cells and are able to induce IFN-γ production, although IL-23 has a unique and prominent role in IL-17 production (9).

Recently, patients have been identified with genetic IL-12/23Rβ1 or IFN-γ receptor (IFN-γR) deficiencies. These individuals have an impaired capacity to produce or respond to IFN-γ, respectively, and often are unusually susceptible to severe infections with weakly pathogenic mycobacteria and salmonellae, but not viral pathogens (3, 12). These patients mostly have normal responses to childhood vaccinations but can develop disseminating disease due to Mycobacterium bovis BCG following live M. bovis BCG vaccination (3, 12). Common viruses like influenza viruses cause worldwide epidemics of respiratory illnesses. CD4⁺ T cells are important in controlling influenza A virus infection, since the induction of antibodies specific for hemagglutinin is dependent on CD4 T-cell help. CD4 T cells also drive the induction and expansion of cytotoxic T cells against such viral pathogens (11). Influenza virus vaccination effectively protects individuals against serious complications through induction of humoral and cellular responses (5). Patients with defects in the IL-12/23/IFN-γ axis provide an interesting model to study the in vivo induction of cellular and humoral immune responses against influenza virus in the absence of molecularly defined components of this essential axis in the human cellular immune response. Herein, we report the induction of humoral and cellular responses following immunization of IL-12/23Rβ1-deficient patients, partial IFN-γR1 (pIFN-γRI)-deficient patients, and healthy controls with an influenza virus vaccine.

MATERIALS AND METHODS

Vaccination protocol. All individuals provided written informed consent to participate in the study. The protocol was approved by the Medical Ethical Board of LUMC (protocol no. P05.117).

Individuals were vaccinated with a trivalent influenza virus subunit vaccine (Influvac, formulation 2001/2002; Solvay Pharmaceuticals BV, Weesp, The Netherlands) containing 15 μg of hemagglutinin of an A/Moscow/10/99-like strain (ResVir 17, a reassortant of A/Panama/2007/99) (H3N2), the A/New Caledonia/20/99 strain (IVR-116) (H1N1), and a B/Sichuan/379/99-like viral strain (B/Guangdong/120/00). Blood samples were collected before, 7 days after, and 28 days after vaccination. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation. Cells were frozen in RPMI 1640 medium (Gibco, Paisley, Scotland) supplemented with 0.04 mM/mL glutamine, 20% fetal calf serum, and 10% dimethyl sulfoxide, stored at −70°C, and transferred to liquid nitrogen the next day until use. Serum samples were stored at −20°C. Samples collected at the different time points were tested in single runs to avoid interexperimental variation.

Human subjects. Patient A was a 30-year-old female who had a heterozygous recessive IL12Rβ1 gene mutation at nucleotide position 94 (C→T) leading to a...
Lymphocyte stimulation test and cytokine production. The lymphocyte stimulation test was performed as described previously (4). In brief, PBMCs (1.5 × 10^6 cells/well) were incubated with antigen (Influvac 2001/2002), control allantoic fluid, or phytosphagglutinin (Murex Biotech Ltd., Dartford, United Kingdom). After 72 h, supernatants were harvested before 0.5 μCi/well of [3H]thymidine was added to determine proliferation (expressed as mean counts per minute of triplicate cultures). Cytokine production was determined by enzyme-linked immunosorbent assay for IFN-γ (U-CyTech, Utrecht, The Netherlands), IL-12 (U-CyTech), and IL-10 (Biosource, Etten-Leur, The Netherlands).

To determine the contribution of IL-12/23 and IL-18 to IFN-γ production, PBMCs were stimulated in the presence or absence of neutralizing antibodies against IL-12/23 (1 μg/ml) (a generous gift from G. Trinchieri) or IL-18 (0.1 μg/ml) (a generous gift from M. Kurimoto). The antibody against IL-12/23 recognizes the IL-12/23 p40 chain and inhibits both IL-12 and IL-23 bioactivity. ELISPOT assay for single-cell IFN-γ release. The number of influenza virus-specific, IFN-γ-producing T cells was enumerated by IFN-γ enzyme-linked immunosorbent assay (ELISPOT). The ELISPOT assay was performed as described previously (4) using an IFN-γ-specific antibody pair (MabTech, Stockholm, Sweden). In brief, PBMCs (2 × 10^5 cells/well) were incubated in the presence of antigen in precoated MultiScren nitrocellulose flat-bottomed 96-well plates (Millipore, Bedford, MA). After 24 h, spots were visualized and plates were analyzed with a Zeiss Asioplan 2 microscope and KS ELISPOT software (Carl Zeiss Vision, Hallbergmoos, Germany). Samples were tested in six replicates, and results were expressed as the mean number of influenza virus-specific IFN-γ-producing cells per million cells.

HI test. Influenza virus strains were propagated in 11-day-old embryonated chicken eggs. Hemagglutination inhibition (HI) tests were performed in duplicate (13) with turkey erythrocytes and four hemagglutinating units of virus. Ferret sera raised against the test antigens were used as positive controls. All sera of individual study subjects were tested simultaneously. For statistical analysis, a titer of 5 was arbitrarily assigned to sera with a titer of <10.

Statistics. Basic descriptive summary statistics were used to display various parameters. Data for proliferative responses, IFN-γ production, number of IFN-γ-specific T cells, and antibody titers were normalized by log transformation. Next, to determine whether the controls and two patient groups differed significantly in the lognormal-distributed dependent variables, a one-way multiple analysis of variance was performed (SPSS 14; SPSS, Inc., Chicago, IL), taking groups (patients and controls) and time points in relation to vaccination (day 0, 7, and 28 after vaccination) as preset independent variables. A P value of <0.05 was taken as an indication of statistical significance.

RESULTS

T-cell responses against the influenza virus vaccine. To investigate whether protective humoral and cellular immune responses, and IFN-γ production in particular, could be induced in the absence of normal IL-12/23/IFN-γ axis signaling, we vaccinated with the seasonal influenza virus subunit vaccine two patients with complete IL-12/23Rβ1 deficiency, two patients with pIFN-γRI deficiency, and five healthy controls. Vaccine-specific T-cell responses were examined in cryopreserved PBMCs collected before, 7 days after, and 28 days after influenza virus vaccination. Proliferative responses and production of IFN-γ, IL-10, and IL-13 were determined in parallel in the same cultures, while the number of influenza virus-specific, IFN-γ-producing T cells was enumerated by IFN-γ ELISPOT.

When patients and controls were analyzed together, a significant increase was observed after vaccination in both the influenza virus-specific PBMCs’ proliferative responses and the number of influenza virus-specific, IFN-γ-producing T cells measured by ELISPOT compared to values before vaccination (P values of 0.031 and 0.014, respectively). The PBMCs’ proliferative responses and influenza virus-specific IFN-γ production were highly correlated (r = 0.72; P < 0.001) (Fig. 1, inset). Of note, PBMC proliferation in patient C at day 0 and in control individual 4 at day 28 was low (Fig. 1A) despite the significant responses measured by ELISPOT (Fig. 1B), which is likely more sensitive. Vaccine-specific responses typically peaked 7 days after vaccination, except for patient B, whose responses were highest 28 days after vaccination. The vaccination-induced increase in influenza virus-specific T-cell proliferation and the number of influenza virus-specific, IFN-γ-producing cells in the controls exceeded those in the patients.

Further analysis showed that the difference between patients and controls was due to lower responses in patients with the dominant negative pIFN-γRI mutation (P values were 0.057 for proliferative responses and <0.01 for ELISPOT responses compared to controls) rather than a lower response in patients with IL-12Rβ1 deficiency. In the latter group, the PBMCs’ proliferative responses and number of influenza virus-specific, IFN-γ-producing T cells were slightly but not significantly lower than in controls (P values of 0.47 and 0.11, respectively). IL-12Rβ1-deficient patients are also impaired in their response to IL-23, which may affect the development of optimal memory T-cell responses (6, 12).

The parents of patient B, who are heterozygous for the relevant IL12RB1 gene mutation, had normal responses in all assays (Fig. 1).

Further experiments showed that IL-13 and IL-10 production was low in all individuals, suggesting that the lower IFN-γ production in patients is not associated with high levels of IL-10 or enhanced Th2 responses (levels of IL-13 were all <80 pg/ml, and levels of IL-10 were all <30 pg/ml; data not shown). The levels of IL-4, IL-5, IL-2, and tumor necrosis factor could be determined in a sample set of three individuals (patient A and controls 1 and 2) but showed no differences between the patient and the controls and no relation to vaccination. The levels of IL-4 in vaccine-stimulated cultures were all below 10 pg/ml, the levels of IL-5 were between 20 and 50 pg/ml, and the levels of IL-2 were between 40 and 90 pg/ml. The levels of tumor necrosis factor in the same supernatants of vaccine-antigen-stimulated PBMC cultures before, 1 week following, and 1 month following vaccination were as follows: 151, 280, and 263 pg/ml for patient A; 53, 128, and 20 pg/ml for control 1; and 172, 474, and 442 pg/ml for control 2, respectively. Collectively, these data show that despite their genetic IL-
12/23Rβ1 or pIFN-γRI deficiency, individuals with these deficiencies are able to induce a Th1 response upon influenza virus vaccination, although especially patients with pIFN-γRI deficiency were less able to produce IFN-γ than the controls.

**Vaccine-induced antibody titers.** Serum antibody titers against the individual influenza virus vaccine strains H3N2, H1N1, and B were determined using an HI test. Overall, titers against the three vaccine components increased significantly after vaccination (for all components, *P* < 0.04) and did not differ between patients and controls (*P* > 0.40) (Table 1). In all cases but one, levels in patients and controls reached protective titers (≥40) after vaccination against each of the three vaccine components. Only (IL-12/23Rβ1 deficient) patient A did not reach a protective antibody titer against H3N2, but this individual had adequate titers against the other components in the trivalent vaccine, i.e., H1N1 and B.

Thus, these findings are in agreement with the above T-cell data, in which patients showed a significant, albeit overall lower, increase in T-cell responses upon vaccination compared to the healthy controls. The HI test only determines the magnitude of the antibody response; we did not investigate the qualitative properties of these antibodies, including their isotypes.
Role of IL-12/23 and IL-18 in anti-influenza immunity. Finally, we wanted to investigate the role of IL-12/23 and IL-18—which acts in synergy with IL-12/IL-23 but can also induce IFN-γ production by itself—in the influenza virus-induced IFN-γ response. We therefore tested whether neutralizing antibodies against IL-12/23 p40 and IL-18 could inhibit vaccine-specific IFN-γ production. When control PBMCs from the time point 7 days postvaccination were stimulated with the influenza virus vaccine, vaccine-induced IFN-γ secretion was not inhibited by neutralizing antibodies against IL-12/23 p40 or IL-18 (Fig. 2). This shows that IFN-γ production elicited by the influenza virus vaccine is IL-12/23 and IL-18 independent. In contrast, however, IFN-γ production induced by *Mycobacterium avium* sonicate from the same PBMC was inhibited almost completely by anti-IL-12/23 p40 antibodies and to a lesser extent by anti-IL-18 alone, thus confirming the strong IL-12/23 dependency of antimycobacterial IFN-γ responses. These data indicate that whereas IL-12/23 plays a major role in IFN-γ production against *M. avium*, it seems redundant in the IFN-γ response against viral antigens in the influenza virus vaccine.

**TABLE 1. Serum antibody titers against the individual influenza virus vaccine strains**

| Human subject | H3N2 | | | H1N1 | | | B | | |
|----------------|------|------|------|------|------|------|------|------|------|
|                | Titer | Sign. increase | Prot. titer | Titer | Sign. increase | Prot. titer | Titer | Sign. increase | Prot. titer |
| Control        | Day 0 | Day 28 | Prot. titer | Day 0 | Day 28 | Prot. titer | Day 0 | Day 28 | Prot. titer |
| 1              | 10    | 40    | Yes     | 80    | 80    | No     | 80    | 1,920 | Yes   |
| 2              | 20    | 40    | No      | 80    | 160   | No     | 80    | 1,280 | No    |
| 3              | 320   | 640   | No      | 10    | 320   | Yes    | 40    | 320   | Yes   |
| 4              | 10    | 240   | No      | 10    | 240   | Yes    | 10    | 1,920 | Yes   |
| 5              | 20    | 80    | Yes     | 40    | 320   | Yes    | 40    | 240   | Yes   |
| Patient        | 1,280 | 480   | No      | 320   | 640   | No     | 320   | 320   | No    |
| A              | <10   | <10   | No      | 60    | 80    | No     | 60    | 80    | No    |
| B              | <10   | <10   | No      | 20    | 120   | Yes    | 20    | 120   | Yes   |
| C              | 1,280 | 640   | No      | 40    | 120   | Yes    | 40    | 120   | Yes   |
| D              | 10    | 240   | Yes     | 10    | 1,920 | Yes    | 10    | 1,920 | Yes   |
|                | 60    | 80    | No      | 60    | 80    | No     | 60    | 80    | No    |

*Sign., significant; Prot., protective.*

DISCUSSION

The main finding of the present study is that patients with genetic defects in the IL-12/IL-23/IFN-γ axis (complete genetic IL-12/23RI deficiency or dominant negative pIFN-γRI deficiency) can elicit significant Th1 responses upon influenza virus vaccination, albeit to a somewhat lower extent than most healthy controls. Importantly, the Th1 response induced by the influenza virus vaccine was found to be independent of IL-12/23, which contrasted sharply with mycobacterium-specific T-cell responses, which were strongly IL-12/23 dependent (3). Of clinical relevance, since patients with genetic defects in the IL-12/23/IFN-γ axis can elicit significant Th1 responses upon influenza virus vaccination, vaccination of these individuals may be of benefit in preventing influenza-related complications, including bacterial superinfection.

IFN-γ plays an important role in controlling influenza virus infections in humans. Elderly people produce lower IFN-γ levels after vaccination and display lower T-cell responses, and this may constitute one of the determinants that make this group more susceptible to symptomatic influenza virus infection. In mice, a protective role of IFN-γ in recall responses to influenza virus has been demonstrated as well: IFN-γ knockout mice have a reduced survival rate upon challenge with influenza virus after vaccination compared to wild-type mice and are unable to clear the virus completely (2). It is likely that patients with pIFN-γRI deficiency can sufficiently respond to IFN-γ to control viral infection (8). Various IFN-γ-dependent cellular effector mechanisms are affected to different extents in pIFN-γRI-deficient patients (7). Selective impairment in killing of certain but not all bacteria or viruses may thus explain these patients’ resistance to most viral infections.

The role of IL-12 in influenza virus infection is less clear. Our results indicate that vaccine-specific IFN-γ production is IL-12/23 independent and also largely IL-18 independent, whereas mycobacterium-induced IFN-γ production is highly IL-12/23 dependent. This is in agreement with the observation that human macrophages do not produce IL-12 and IL-23 after infection with influenza virus (14). Instead, they produce IFN-α/β and IL-1β, which are able to induce IFN-γ production as well (14, 15). Moreover, several reports have suggested that IL-18 may be important in the induction of IFN-γ in influenza virus infection (14, 15). This latter observation is not directly

**FIG. 2.** The role of endogenous IL-12/23 and IL-18 in response to the influenza virus vaccine (vaccin) compared to *M. avium* (Mav.). PBMCs of three healthy controls (contr.) (controls 1, 2, and 4) collected 7 days postvaccination were stimulated with the influenza virus vaccine or *M. avium* sonicate in the presence of neutralizing anti-IL-12/23 antibody, neutralizing anti-IL-18 antibody, or an isotype-matched control antibody. After 3 days, IFN-γ production in the supernatant was measured. Results are the means of the responses for the three control individuals tested and are expressed as percent IFN-γ response compared to stimulation with antigen in the presence of the control antibody. mAb, monoclonal antibody; α, anti.
supported by our results, which fail to show an important role for IL-18 in IFN-γ production in response to the influenza virus vaccine. One explanation for this discrepancy might be that vaccination with inactivated virus induces other cytokine responses than a wild-type viral infection. Another, not mutually exclusive, explanation is that the influenza virus vaccine-specific responses studied here are likely to be secondary in nature, at least in part, since several individuals had previously received influenza virus vaccinations. Nevertheless, primary responses are likely to be involved, also since cross-reactivity between the different influenza virus strains included in the yearly vaccines is limited, such that vaccine-induced responses will be primed also toward new epitopes unique to each vaccine. In any case, IL-12 does not seem to play a critical role in the clearance of influenza virus infection in mice, and IL-12 may be needed only for early production of IFN-γ by NK cells, but not for the late IFN-γ production by T cells (10, 16). Thus, IL-12 does not seem to play an essential role in adaptive immunity and the induction of immune protection against influenza virus infection in mice and humans.

In conclusion, influenza virus vaccination induces significant IL-12/23-independent IFN-γ production and sufficient humoral protection in IL-12/23R1- and pIFN-γRI-deficient individuals. Thus, seasonal influenza vaccination of these individuals should be considered to prevent influenza-related complications in these immunocompromised individuals.

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