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### Measles Virus Infection in Adults Induces Production of IL-10 and Is Associated with Increased CD4<sup>+</sup>CD25<sup>+</sup> Regulatory T Cells<sup>1</sup> **FREE**

Xue-lian Yu; ... et. al

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# Measles Virus Infection in Adults Induces Production of IL-10 and Is Associated with Increased CD4<sup>+</sup>CD25<sup>+</sup> Regulatory T Cells<sup>1</sup>

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Despite steady progress in elimination of measles virus globally, measles infection still causes 500,000 annual deaths, mostly in developing countries where endemic measles strains still circulate. Many adults are infected every year in China, with symptoms more severe than those observed in children. In this study, we have used blood samples from adult measles patients in Shanghai and age-matched healthy controls to gain an understanding of the immune status of adult measles patients. IFN- $\alpha$  mRNA was reduced in patient PBMC compared with healthy controls. In contrast, gene expression and plasma production of IL-2, IL-10, and IFN- $\gamma$  were elevated in patient blood. A similar cytokine profile was observed at early times when cultured PBMC were infected with a clinical isolate of measles virus. In contrast to previous studies in pediatric patients, we did not find a reduction in total CD4<sup>+</sup> and CD8<sup>+</sup> T cells in patient PBMC. Interestingly, we found that CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup> regulatory T cells were significantly increased in patient PBMC compared with controls. Using intracellular cytokine staining we also show that the measles virus induces IL-10-producing CD14<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup> cells in PBMC. Our results show that adult measles patients in the acute phase of the disease have a mixed Th1/Th2 type response, accompanied with severe immunosuppression of both innate and adaptive responses including suppression of type I IFN. Both regulatory T cells and plasma IL-10 may contribute to the immunosuppression. *The Journal of Immunology*, 2008, 181: 7356–7366.

**M**easles virus (MV)<sup>5</sup> is a highly contagious respiratory pathogen that causes systemic disease; most individuals recover with lifelong immunity to MV. Enormous progress toward measles elimination has been made worldwide, including China, in large part due to the availability of a safe and effective vaccine (1–5). However, measles infections still cause 500,000 deaths annually, mostly due to subsequent opportunistic infections associated with immunosuppression (3). Widespread vaccination may lead to an increase in the average age at infection (2); recent data from China (1) and other countries (6, 7) support this hypothesis. With more infections in higher age groups, in-

creased incidence of severe disease and even fatalities may follow (8). A large proportion of adult measles patients in China present with diarrhea, pneumonia, and liver dysfunction (9–11) and may have immune responses different from those in children.

MV infection is accompanied by severe suppression of both innate and adaptive immune response that may last for months (12) and is probably caused via multiple mechanisms (13). MV reduces proliferation of innate effector cells, including macrophages, NK cells, and neutrophils in cultured PBMC (14), in measles infection in children (15, 16), and in animal models (17, 18). MV infection also results in reduced lymphocyte numbers in children (19), in young adults (20), in cultured PBMC (21), and in animal models (17).

Following MV infection in children, an early Th1 response characterized by high levels of IFN- $\gamma$  and IL-2 is quickly succeeded by a prolonged Th2 or mixed Th1/Th2 response characterized by IL-4, IL-13, and TNF- $\alpha$ , with elevated IL-10 levels persisting (19, 22). Elevated levels of plasma IL-10 probably contribute to impaired cellular immunity and depressed hypersensitivity responses following MV infection (19). The primary cellular source of IL-10 in MV infection is not clear, although various cell types, including neutrophils and monocytes (19, 23), have been suggested. Interestingly, IL-5 is either absent (24) or decreased during MV infection (19), probably reflecting varying levels of baseline IL-5 and eosinophilia. Cells infected with clinical strains of MV, but not vaccine strains, display nearly complete suppression of IFN- $\alpha$ -induced antiviral state probably due to the activities of proteins encoded by the MV P gene (25).

Regulatory T cells (Treg) have gained recognition in recent years as important mediators of immunosuppression in disease, but they have not been investigated in MV infection to date. Treg have been shown to suppress T cell responses leading to reduced viral

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<sup>5</sup> Abbreviations used in this paper: MV, measles virus; MOI, multiplicity of infection; CBA, cytometric bead array; Treg, regulatory T cell; IQR, interquartile range.

Table I. PCR primers and conditions

Cytokine	Forward Primer	Reverse Primer	Product Length in bp
IL-2 <sup>a</sup>	ATGTACAGGATGCAACTCCTGTCTT	CAGTTTCGTAGTAGAGTTGTGATTG	458
IL-4 <sup>b</sup>	ATGGGTCTCACCTCCCAACTGCCT	TACTCTCTCTCTTTATAAGTTTCACAAGC	456
IL-10 <sup>b</sup>	ATGCCCAAGCTGAGAACCAAGACCCA	TACTGTTACTTCTATGCTTTG	249
IL-5 <sup>a</sup>	TCATCACGCCAAGGAACCTCT	TGACGAGCAATGAGACGATG	229
IFN- $\gamma$ <sup>b</sup>	ATGAAATATACAAGTTATATCTTGGCTTT	TACGACAAAGCTCCAGCTTCTCGTAG	494
TNF- $\alpha$ <sup>a</sup>	GGATGAACACGCCAGTCGCC	CGAGTGACAAGCCCGTAGCC	468
IFN- $\alpha$ <sup>c</sup>	AATCTCTCCTTTCTCTCCTG	ACACGGACCCTCCAACAGTCT	364
$\beta$ -actin <sup>d</sup>	GTACCCCATCGAGCACGGCAT	TGGGTCATCTTCTCGCGTT	180

<sup>a</sup> PCR: 30 cycles of 45 s at 94°C, 45 s at 50°C, 1 min at 72°C.

<sup>b</sup> PCR: 35 cycles of 1 min at 94°C, 1 min at 58°C, 1 min at 72°C.

<sup>c</sup> PCR: 30 cycles of 20 s at 95°C, 25 s at 65–50°C, 40 s at 72°C and 10 cycles of 20 s at 95°C, 25 s at 50°C, 1 min at 72°C.

<sup>d</sup> PCR: 30 cycles of 45 s at 94°C, 45 s at 53°C, 1 min at 72°C.

clearance and establishment of conditions favorable for opportunistic infections (26); additionally, Treg are able to secrete IL-10 in disease states and may have a role in the immunosuppression observed in MV infection.

In the current study, we show that analogous to the observations in children, innate responses were suppressed in adult acute measles patients, and that IL-10, IL-2, and IFN- $\gamma$  were elevated. In contrast, total T lymphocytes were not reduced in the adult measles patients in our study, although monocyte/macrophage populations were significantly decreased. We show for the first time that Treg (CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup>) are increased in adult measles patients. Using intracellular cytokine staining, we also show that CD4<sup>+</sup>CD25<sup>+</sup> T cells and CD14<sup>+</sup> monocyte/macrophages contribute to the production of IL-10.

## Materials and Methods

### Subjects

Samples from 40 measles patients and 25 healthy subjects were used in this study. Patients admitted to Shanghai Public Health Clinical Center during the measles epidemic seasons of 2006 and 2007 were enrolled in this study with consent. A blood sample was collected at hospital admission, and a self-administered questionnaire (including demographic information, vaccination history, and clinical symptoms) was filled out by the patients with help from attending clinicians. Blood samples from age- and sex-matched anonymous adult blood donors reporting to the Shanghai Red Cross blood center were used as healthy controls; measles infection and vaccination history were not recorded.

### PBMC isolation and cryopreservation

Plasma and PBMC were separated from venous blood by standard Ficoll-Hypaque (Accuspin System-Histopaque; Sigma-Aldrich) centrifugation. Heparinized blood was mixed with 2 vol of prewarmed PBS (pH 7.4) and subjected to Ficoll-Hypaque density gradient centrifugation at 800  $\times$  g for 15 min. Plasma samples were aliquoted and stored at  $-80^{\circ}\text{C}$  until needed. PBMC were also collected, transferred to a fresh tube, and washed twice in prewarmed PBS. Cells were counted, resuspended in RPMI 1640 (Sigma-Aldrich) freezing media containing 10% DMSO (Sigma-Aldrich) and 10% FBS (Invitrogen), frozen at  $-80^{\circ}\text{C}$  overnight, and stored in liquid nitrogen until needed.

### Cell thawing after cryopreservation

Cryopreserved cells were thawed by a modification of a previously published procedure (27). Cells were quickly thawed and diluted for a period of 5 min with a 10-fold excess of 10% FBS in RPMI 1640 prewarmed to room temperature. After centrifugation at 300  $\times$  g for 5 min, the cell pellet was gently resuspended and washed with an equal volume of PBS prewarmed to room temperature. After centrifugation, cells were resuspended in RPMI 1640 medium containing FBS and incubated at 37°C in 5% CO<sub>2</sub> for 2 h before use.

### Virus and cell culture

MV was isolated from one of the patient samples using standard protocols and was used throughout the study (28, 29). In brief, a sample of patient

urine (50–100 ml) was centrifuged (1500 rpm, 30 min) within 4 h of collection, the pellet resuspended in virus transport medium (DMEM containing 2% FBS, with 1000 U/ml penicillin and 1000  $\mu\text{g}/\text{ml}$  streptomycin), and used to inoculate semiconfluent Vero-SLAM cell monolayers grown in DMEM containing 10% FBS. After adsorption at 37°C for 2 h, inoculum was replaced with fresh DMEM containing 2% FBS and incubated at 37°C in 5% CO<sub>2</sub> atmosphere. Cells were observed for cytopathic effect daily until extensive cytopathic effect (50–75% monolayer affected) was observed. Cells were lysed by freeze-thaw cycles and cellular debris removed by centrifugation at 6000 rpm for 10 min. The supernatants were passed through a 0.22- $\mu\text{m}$  filter, aliquoted, and stored at  $-80^{\circ}\text{C}$  as virus stock. A control uninfected Vero-SLAM lysate was prepared similarly. Multiplicity of infection (MOI) was based on the viral titer of the stock preparation as determined by standard assays (30).

PBMC were cultured in RPMI 1640 supplemented with 100 U/ml penicillin-streptomycin (Sigma-Aldrich) and 10% FBS at 10<sup>6</sup> cells/well in triplicate wells of 24-well microtiter plates for all assays. MV or control Vero-SLAM lysate was added at indicated MOI, and the cultures were incubated at 37°C in 5%. The cell culture medium was replaced with fresh RPMI 1640 containing 10% FBS 2 h later, followed by incubation for indicated times. Cell supernatants were collected and cells lysed with RNazol (Biotex Laboratories); both supernatants and cell lysates were stored at  $-80^{\circ}\text{C}$  before use.

### Measles IgM Ab assay

Measles IgM Ab was assayed in all serum samples using a commercial measles IgM enzyme-linked immunosorbent assay kit (ELISA capture assay, MV IgM Ab diagnostic kit; Haitai Bio-product Company) as recommended by the manufacturer. All kit components were prewarmed to room temperature, plasma was diluted 1/100 with dilution buffer, and 100  $\mu\text{l}$  of each diluted sample (or control) was used per well

Table II. General and clinical information of study subjects

	Healthy Controls	Measles Patients
Gender		
Male	52% (13/25)	50% (20/40)
Female	48% (12/25)	50% (20/40)
Age		
Median	36	35
Range	20–61	18–62
Day of sample collection post-rash onset		
Median		4.5
Range		0–20
Clinical symptoms		
Koplik spot		70% (28/40)
Coryza		87.5% (35/40)
Conjunctivitis		77.5% (31/40)
Bronchitis/pneumonia		57.5% (23/40)
Vomit/diarrhea		57.5% (23/40)
Liver dysfunction		20% (8/40)

in duplicate. All subsequent steps were performed at 37°C. After 1-h incubation, wells were washed three times followed by the addition of 50  $\mu$ l of HRP conjugate solution to each well and incubation for 1 h. Wells were washed and incubated for 10 min in the dark with the substrate solution. Reaction was stopped and absorbance at 450 nm estimated within 5 min. A blank sample containing only the substrate and stop reagents was also included and used to calibrate the plate reader. Any sample with value of  $OD_{\text{sample}}/OD_{\text{negative control}} \geq 2.1$ , was defined as measles IgM Ab positive. A confirmed case of measles infection was defined as one having tested positive for measles IgM in an individual who received no measles vaccination within the previous month.

### IFN- $\alpha$ ELISA

Plasma IFN- $\alpha$  production was detected in samples using a commercial IFN- $\alpha$  (human) Serum Sample ELISA kit (PBL Biomedical Laboratories), using the high sensitivity protocol according to the manufacturer's recommendations; IFN- $\alpha$  levels were determined by comparison with a standard curve.

### Cytometric bead array (CBA)

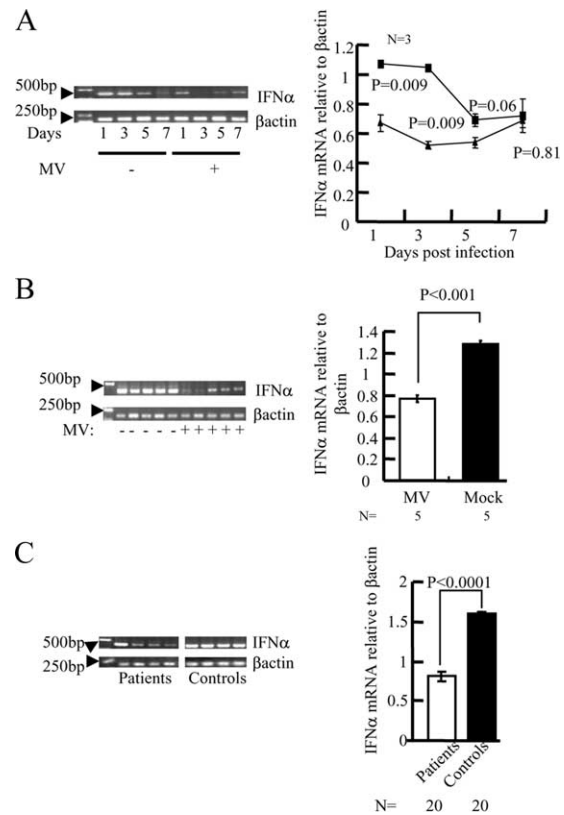
CBA for Th1/Th2 cytokines in plasma or culture supernatants were performed as per manufacturer's recommendations (Falcon; BD Biosciences). Six bead populations with distinct fluorescence intensities coated with capture Abs specific for IL-2, IL-4, IL-5, IL-10, TNF- $\alpha$ , and IFN- $\gamma$  were mixed and added to each assay tube, followed by PE detection reagent and cell culture supernatant or plasma. Mixtures were incubated at room temperature for 3 h in the dark, washed, and then analyzed using flow cytometry. The data were analyzed using the BD CBA Analysis software. The individual cytokine concentration of each test sample was calculated by reference to a standard curve. Assay sensitivity: IL-2, 2.6 pg/ml; IL-4, 2.6 pg/ml; IL-5, 2.4 pg/ml; IL-10, 2.8 pg/ml; TNF- $\alpha$ , 2.8 pg/ml; IFN- $\gamma$ , 7.1 pg/ml.

### FACS analysis

Cryopreserved cells were thawed as described above and cultured in RPMI 1640 containing 10% FBS for 2 h. Cells were harvested from cultures, washed with ice cold PBS containing 2% FBS, and resuspended in 100- $\mu$ l staining buffer (PBS containing 2% FBS), followed by a staining mixture consisting of anti-CD3-PerCP, anti-CD14-allophycocyanin, anti-CD4-FITC, anti-CD25-PE, and anti-CD127-PE-Cy7; or anti-CD3-PerCP, anti-CD4-FITC, and anti-CD8-PE, using 10  $\mu$ l (Abs obtained from BD Biosciences) or 20  $\mu$ l (Abs obtained from eBioscience) of each mAb. Control staining combinations included mixtures of IgG1 conjugated to each of the above fluorochromes or IgG2a-allophycocyanin. Anti-CD3-PerCP and anti-CD14-allophycocyanin were purchased from BD Pharmingen, and anti-CD4-FITC, anti-CD25-PE, anti-CD8-PE, and CD127-PE-Cy7 were purchased from eBioscience. After 30-min incubation at room temperature in the dark, cells were collected by centrifugation at  $300 \times g$  for 5 min, washed twice, resuspended in 500- $\mu$ l staining buffer, and then analyzed. Cells of the monocyte/macrophage lineage were identified after gating on global peripheral blood cells. CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup> T cells were identified after gating on CD3<sup>+</sup> cell blasts. Flow cytometric analysis was done using a FACSAria with FACSDiva Software (BD Biosciences) and 30,000 events were acquired for each measurement. The data are presented as absolute number of respective cells in 30,000 PBMC unless otherwise stated.

### Intracellular cytokine staining

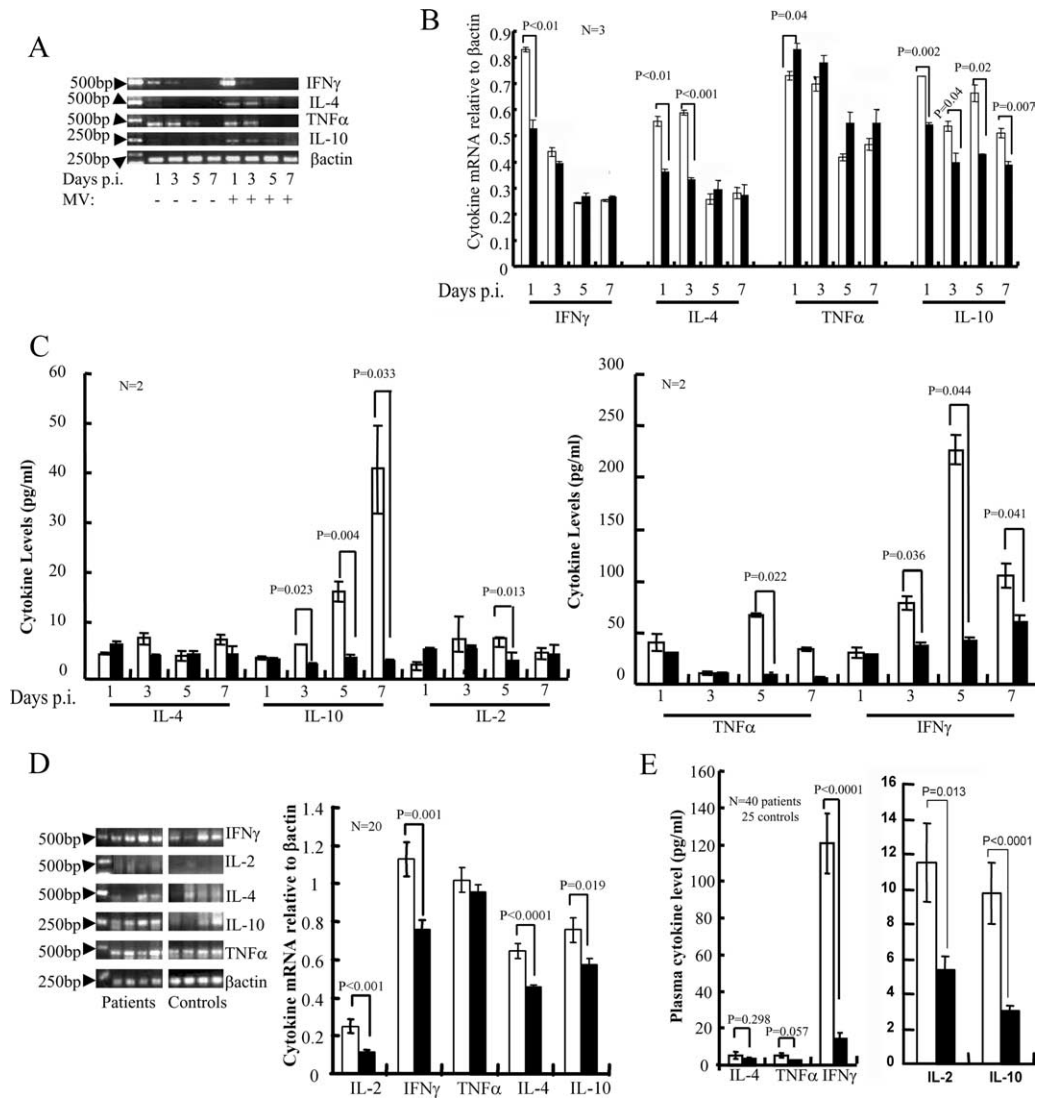
PBMC from healthy donors were cultured in RPMI 1640 containing 10% FBS at  $10^6$  cells/tube and incubated with MV or Vero-SLAM lysate at MOI = 1. Inoculum was replaced with fresh RPMI 1640 containing 10% FBS after 2-h incubation. Cells were cultured for 3 days followed by treatment with PMA (10 ng/ml; Sigma-Aldrich), ionomycin (1  $\mu$ g/ml; Sigma-Aldrich), and brefeldin A (10  $\mu$ g/ml; eBioscience) for 3 h. Cells were surface labeled with a staining mixture containing anti-CD3-PerCP, anti-CD4-FITC, and anti-CD25-PE, or with anti-CD14-PE alone followed by permeabilization for 15 min with 100  $\mu$ l of permeabilization buffer (eBioscience), and incubation in anti-IL-10-Alexa Fluor 647 (eBioscience) for 30 min. Four-color FACS acquisition and analysis were performed as described above. PBMC isolated from patient blood were cultured for 2 h in RPMI 1640 containing 10% FBS and treated with PMA, ionomycin, and brefeldin A, followed by processing for intracellular cytokine staining as for PBMC from healthy donors.



**FIGURE 1.** IFN- $\alpha$  expression is suppressed in measles infection. **A**, PBMC were isolated from whole blood of healthy controls and infected with a clinical MV isolate (MOI = 1, lanes labeled +) or equivalent volume of Vero-SLAM lysate (lanes labeled -). Cells were collected at indicated times and total RNA extracted. Following reverse transcription with random hexamers, specific PCR was performed for IFN- $\alpha$  (upper panel) and  $\beta$ -actin (lower panel). PCR products were separated by gel electrophoresis and visualized by ethidium bromide staining; the relevant DNA marker is indicated. Gel images such as those shown on the left were analyzed with Image J shareware; data shown on the right have been normalized to  $\beta$ -actin as described in the text. Squares, mock-infected samples; triangles, MV-infected samples. The data represent mean  $\pm$  SEM from three independent experiments; *p* values are indicated. **B**, PBMC from five different healthy controls were treated as in **A** above, cells collected 24 h later, and IFN- $\alpha$  gene expression investigated with RT-PCR as described in **A** above. Open column, MV-infected samples; filled column, mock-infected samples. Statistical difference between the two groups is indicated; *n* = 5. **C**, PBMC were isolated from whole blood obtained from measles patients and age-matched healthy controls, total RNA extracted, and RT-PCR for IFN- $\alpha$  performed as in **A** above. Open column, patient samples; filled column, control samples. Statistical difference between the two groups is indicated; *n* = 20.

### RT-PCR

RNA was extracted from PBMC with RNeasy B followed by reverse transcription using random hexamers and SuperScript II Reverse Transcriptase (Invitrogen) according to manufacturer's protocol. Previously described primer sets (28, 31) were used to amplify specific fragments of IL-2, IL-4, IL-5, IL-10, TNF- $\alpha$ , IFN- $\gamma$ , IFN- $\alpha$ , and  $\beta$ -actin (primers and the cycling parameters used are listed in Table I). PCR products were visualized on a ChemidocXRS imaging system (Bio-Rad) following gel electrophoresis and staining with ethidium bromide; product size was estimated with comparison to a 500-bp marker (Invitrogen). The amount of PCR product relative to  $\beta$ -actin was estimated by measurement of the intensity of the band of PCR product using the shareware ImageJ 1.62. To account for intergel and the considerable interpatient variability, we used a 2-fold normalization system to estimate relative cytokine expression levels. The luminescence index was calculated by dividing the sample luminosity value by the luminosity of the corresponding DNA marker band. The relative ratio was



**FIGURE 2.** Cytokine mRNA expression profile of PBMC treated with or without MV and in measles patients. *A*, PBMC from healthy controls were treated with MV (+) or Vero-SLAM lysate (–) as described in the legend to Fig. 1*A*, and Th1 and Th2 cytokine mRNA expression was determined by RT-PCR. The identity of the PCR products is indicated on the *right*; corresponding DNA size markers are indicated on the *left*. *B*, Gel images such as those shown in *A* above were analyzed with Image J 1.62; data shown have been normalized to  $\beta$ -actin. The data represent mean  $\pm$  SEM of three independent experiments. Open columns, MV-treated samples; filled columns, Vero-SLAM-treated samples. *C*, PBMC from healthy blood donors were treated with MV or Vero-SLAM lysate as described in the legend to Fig. 1*A*, and Th1 and Th2 cytokines were assayed in the culture supernatant using CBA. Open columns, MV-treated samples; filled columns, Vero-SLAM-treated samples. Statistical differences are indicated; data represent means  $\pm$  SD of four data points from two independent experiments. *D*, PBMC from patients and healthy controls were treated and Th1 and Th2 cytokine mRNA expression determined by RT-PCR as described in the legend to Fig. 1*C*. Gel images from four patient and four control samples are representative of a total 20 patients, and 20 control samples are shown on the *left*; images were analyzed as in *B* above and resultant histogram is shown on the *right*. Open columns, patient samples; filled columns, control samples; statistical differences are indicated;  $n = 20$ . *E*, Plasma was isolated from whole blood of measles patients and healthy controls and Th1 and Th2 cytokines measured using CBA. Open columns, patient samples; filled columns, control samples. Statistical differences are indicated;  $n = 40$  patients or 25 controls.

calculated by dividing the cytokine luminescence index by the corresponding  $\beta$ -actin luminescence index.

*Statistical analyses*

All data were processed by SPSS for Windows version 11.5. Differences in age and gender ratio between patients and healthy donors and cell numbers between patient and healthy donors were analyzed using the nonparametric Kruskal-Wallis  $\chi^2$  test. Differences in cytokine mRNA and plasma production between patients and subjects were analyzed using Student’s *t* test. Differences in cytokine mRNA and protein production between MV- and mock-treated cells were analyzed using paired *t* test. Pearson correlation was used to analyze the relationship between patients’ plasma IL-10 levels and three different peripheral cell numbers followed by regression analysis to fit the data to a curve. Significance was accepted at  $p \leq 0.05$ .

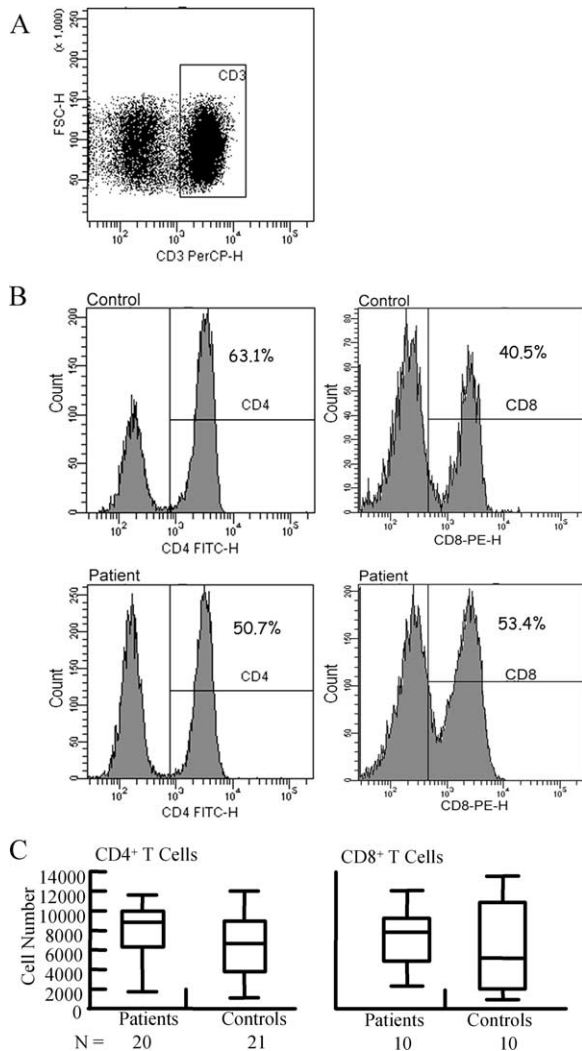
**Results**

*Study subjects*

The general information regarding measles patients and healthy controls, including the clinical symptoms, is listed in Table II. The median age of the patients (35 years; interquartile range (IQR), 28–43 years) did not differ from that of healthy controls (36 years; IQR, 27.25–41 years;  $p = 0.43$ , Kruskal-Wallis  $X^2$  test). About half (50%) of the patients and healthy controls (52%) were male ( $p = 0.97$ , Kruskal-Wallis  $\chi^2$  test). The median number of days of prodrome was two (IQR, 1–3 days), and the median number of days of rash at study entry was 4.5 (IQR, 3–6 days). Seventy

Table III. Plasma IFN- $\gamma$ , IL-2, and IL-10 levels in study subjects

Subject Group	N	IFN- $\gamma$		IL-2		IL-10	
		Median pg/ml (IQR)	<i>p</i> <sup>a</sup> Value	Median pg/ml (IQR)	<i>p</i> Value	Median pg/ml (IQR)	<i>p</i> Value
<3 Days after rash onset	15	122.00 (100.87)	0.0001 <sup>b</sup>	10.10 (7.50)	0.019 <sup>b</sup>	8.64 (6.97)	0.001 <sup>b</sup>
4–7 Days after rash onset	20	84.31 (185)	0.0001 <sup>b</sup>	3.90 (7.56)	0.828	4.61 (4.22)	0.017 <sup>b</sup>
8–14 Days after rash onset	5	60.47 (187)	0.014 <sup>b</sup>	14.93 (26.56)	0.019 <sup>b</sup>	10.70 (38.33)	0.013 <sup>b</sup>
Control	25	9.13 (11.76)		4.97 (5.11)		2.85 (2.02)	

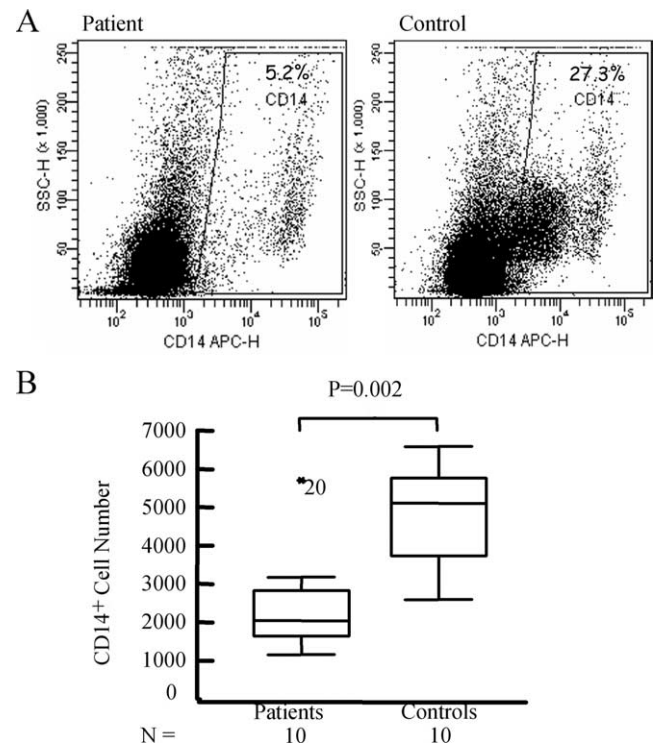
<sup>a</sup> Patients vs controls.<sup>b</sup> Significantly different from controls.

**FIGURE 3.** CD4<sup>+</sup> and CD8<sup>+</sup> T cell populations are not changed in PBMC of measles patients compared with controls. Cryopreserved PBMC from patients and controls were recovered, cultured for 2 h, and stained with a mix of mAbs to CD3, CD4, and CD8 as described in the text. Side scatter (SSC-H, a measure of granularity of the cell) and forward scatter (FSC-H, a measure of cell size) were used to perform the initial gating to select live PBMC. *A*, CD3<sup>+</sup> cells were gated to identify total T cells and to exclude cells of the monocyte lineage; cells not stained with anti-CD3-perCP Abs were used as negative control. *B*, CD4<sup>+</sup> and CD8<sup>+</sup> T cells within the CD3<sup>+</sup> population were analyzed by FACS. Histograms from representative experiments are shown; CD4<sup>+</sup> T cells on the left, CD8<sup>+</sup> T cells on the right; upper histograms represent control PBMC and lower histograms represent patient PBMC. Data are shown as number of cells (count) vs FITC (CD4) or PE (CD8) fluorescence intensity. Cells stained with anti-CD3-perCP Abs alone were used as negative control. Proportion of CD4<sup>+</sup> or CD8<sup>+</sup> cells within the total CD3<sup>+</sup> population is shown as a percentage. *C*, Data obtained from FACS histograms such as shown in *B* above were pooled and are depicted as box and whisker plots. Data shown are medians and percentiles; sample numbers are indicated.

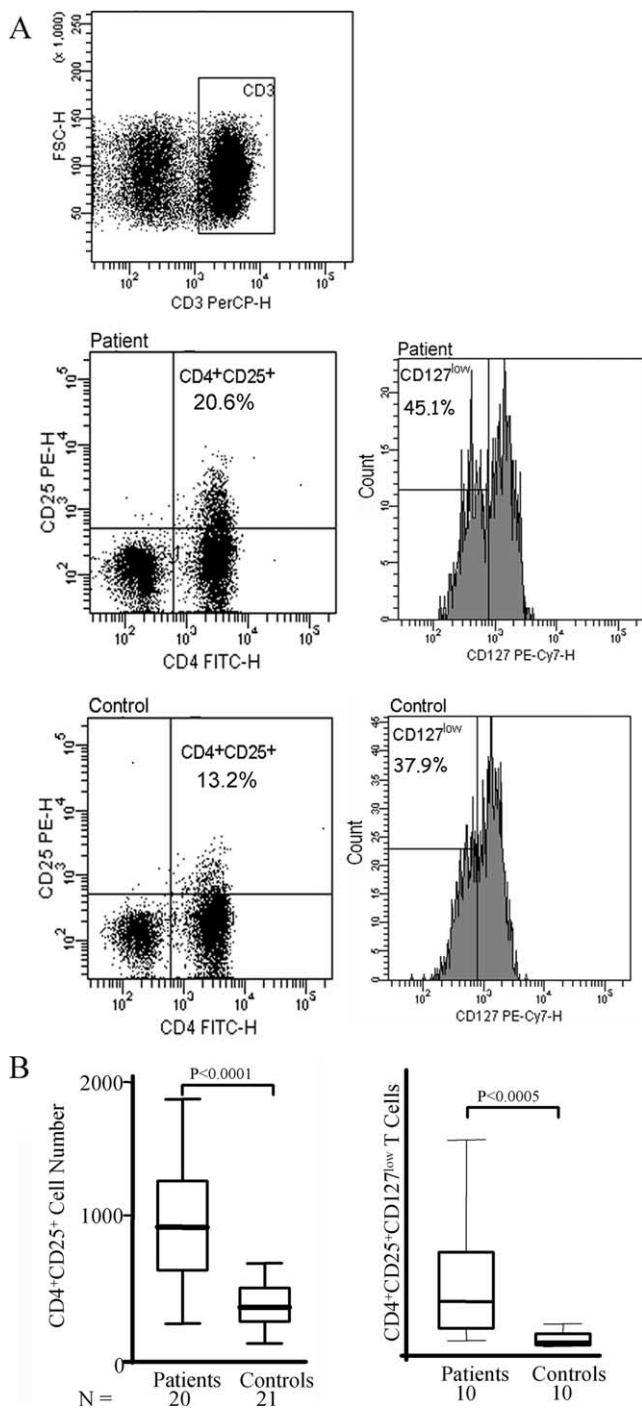
percent (95% Confidence Interval for Mean [95% CI], 52–83%) of patients presented Koplik spot in their mouth mucosa; 77.5% (95% CI, 64–91%) developed conjunctivitis; 57.5% (95% CI, 39–71%), 57.5% (95% CI, 41–74%), and 20% (95% CI, 7–33%) had bronchitis/pneumonia, vomit/diarrhea, and liver dysfunction, respectively. All patients recovered completely.

#### IFN- $\alpha$ is suppressed by MV in vitro and in vivo

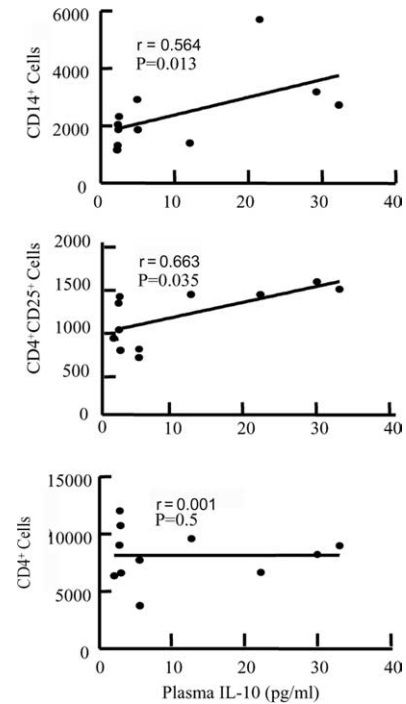
In separate studies, MV has been reported to either induce IFN- $\alpha$  production (32) or suppress IFN- $\alpha$  biosynthesis (25). MV was isolated from urine of one of the patients described above and used to infect control PBMC in cell culture. PBMC were treated with MV (MOI = 1) or Vero-SLAM lysate (mock), and samples (cell lysate



**FIGURE 4.** CD14<sup>+</sup> monocyte/macrophage populations are reduced in measles patients. *A*, Cryopreserved PBMC from patients and controls were recovered, cultured for 2 h, and stained with anti-CD14-allophycocyanin. The total peripheral blood cells were gated as described in the legend to Fig. 3, and a second gate for CD14-expressing cells was set; data are shown as scattergrams of representative patient (left panel) and control (right panel) PBMC. PBMC treated with an isotype-control Ab were used as negative control. Proportion of CD14<sup>+</sup> cells within PBMC is shown as a percentage. *B*, Data obtained from FACS scattergrams such as those shown in *A* above were pooled and are depicted as box and whisker plots. Data shown are medians, percentiles, and outlying values. Statistically significant difference between the two groups and sample numbers are indicated.



**FIGURE 5.** Measles patients' PBMC have increased number of CD4<sup>+</sup>CD25<sup>+</sup> Treg. *A*, Cryopreserved PBMC from patients and controls were recovered and cultured for 2 h, and stained with a mix of mAbs for CD3, CD4, CD25, and CD127 as described in the text. CD3<sup>+</sup> cells were gated as described in the legend to Fig. 3. Side scatter (SSC-H, a measure of granularity of the cell) and forward scatter (FSC-H, a measure of cell size) were used to perform the initial gating. CD3<sup>+</sup> cell population was first analyzed for CD4<sup>+</sup>CD25<sup>+</sup> cells; data are shown for representative patient (upper panel left) and control (lower panel left) PBMC. CD4<sup>+</sup>CD25<sup>+</sup> cells were then analyzed for CD127 expression; data are shown for representative patient (upper panel right) and control (lower panel right) PBMC. Proportion of CD4<sup>+</sup>CD25<sup>+</sup> cells within the CD4<sup>+</sup> population and CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup> cells within the CD4<sup>+</sup>CD25<sup>+</sup> population is shown as a percentage. *B*, Data obtained from FACS scattergrams such as those shown in *A* above were pooled and are depicted as box and whisker plots. Data shown are medians and percentiles. Statistically significant difference between the two groups and sample numbers are indicated.



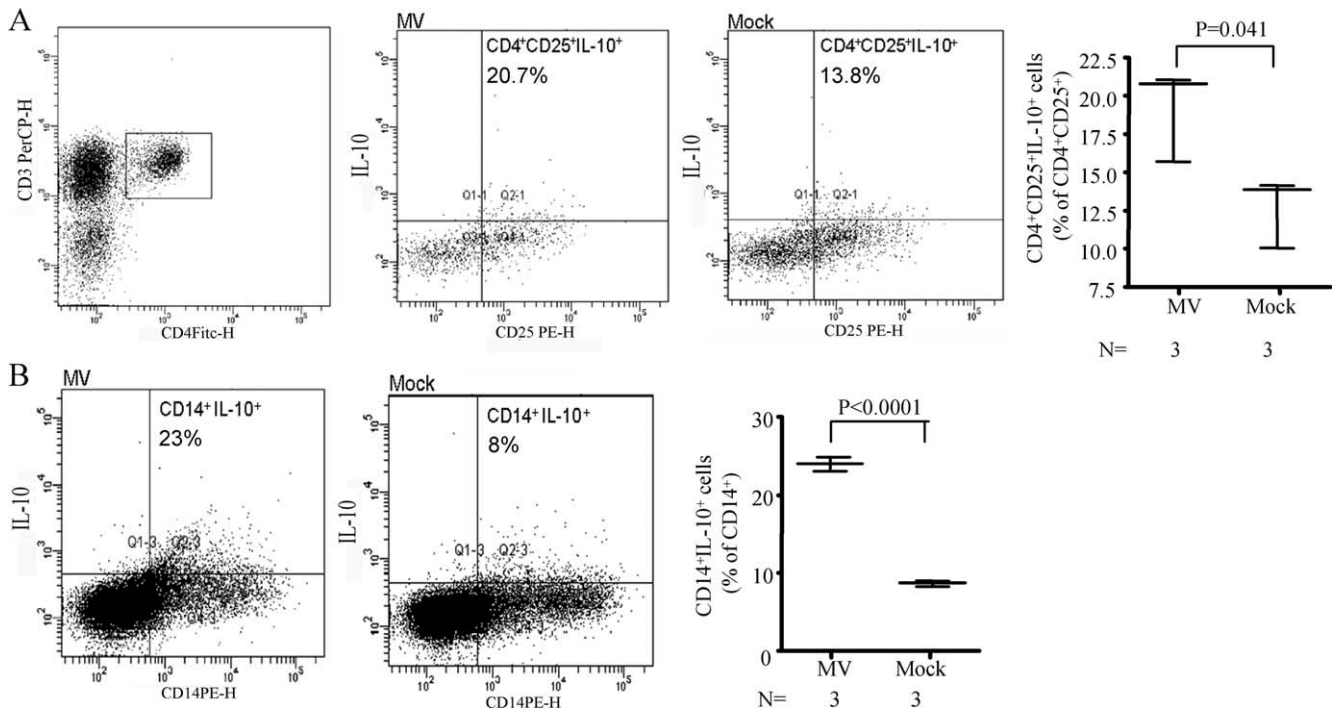
**FIGURE 6.** CD14<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup> cell populations in measles patients are associated with plasma IL-10 levels. Associations of the CD14<sup>+</sup> (upper graph), CD4<sup>+</sup>CD25<sup>+</sup> (middle graph), and CD4<sup>+</sup> (lower graph) cell populations with IL-10 were analyzed by Pearson correlation;  $r$  (coefficient of correlation) and  $p$  values are indicated on respective graphs obtained by regression analysis.

and culture supernatant) were collected at days 1, 3, 5, and 7 after treatment. IFN- $\alpha$  mRNA levels in MV-treated cells remained lower than mock-treated cells at all times and were significantly different on days 1 and 3 ( $p = 0.009$ ; Fig. 1*A*). The observed suppression of IFN- $\alpha$  was not donor specific, because a similar suppression was observed when PBMC from five different control donors were treated with MV and samples analyzed 24 h later ( $p < 0.001$ ; Fig. 1*B*). Our results suggest that IFN- $\alpha$  suppression occurs early in MV infection and is a characteristic of the virus, not the host.

IFN- $\alpha$  mRNA expression was significantly suppressed in patient PBMC compared with healthy controls ( $p < 0.0001$ ; Fig. 1*C*), correlating with undetectable plasma IFN- $\alpha$  levels in patients (data not shown). Our data are in agreement with other studies showing inhibition of IFN- $\alpha/\beta$  by MV, presumably through the actions of V and C proteins (25).

*IFN- $\gamma$  and IL-10 are strongly activated by MV in vitro and in vivo*

Changes in the pattern of Th1- and Th2-type cytokine expression in the course of MV infection have variously been shown to be either a Th1 to Th2 shift (19, 22), a predominantly Th1 state (33), or a mixed Th1/Th2 pattern (6). The cytokine profile induced by treatment of PBMC with MV was investigated. Control PBMC were treated with MV clinical isolate or mock-treated and samples analyzed for IL-4, IL-10, IFN- $\gamma$ , and TNF- $\alpha$  1, 3, 5, and 7 days later. The expression of all cytokines tested peaked at day 1, (Fig. 2, *A* and *B*) coincident with the observed inhibition of IFN- $\alpha$  (see Fig. 1*A*); IFN- $\gamma$ , IL-4, and IL-10 were increased whereas TNF- $\alpha$  was decreased in MV-infected compared with mock-infected samples (IFN- $\gamma$  and IL-4,  $p < 0.01$  vs controls; IL-10,  $p = 0.002$ ; TNF- $\alpha$ ,



**FIGURE 7.** MV treatment up-regulates IL-10-producing CD14<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup> cells in vitro. Control PBMC cultured in RPMI 1640 supplemented with 10% FBS were treated with MV or Vero-SLAM lysate at MOI = 1 for 2 h. Cells were cultured for 3 days followed by incubation with PMA, ionomycin, and brefeldin A for 3 h. A, Cells were surface-labeled with Abs to CD3, CD4, and CD25, permeabilized, and labeled with anti-IL-10-Alexa Fluor 647 Ab. Four-color flow cytometry acquisition and analysis were performed on a FACSaria with FACSDiva software. CD3<sup>+</sup>CD4<sup>+</sup> cells were gated to identify CD4<sup>+</sup> T cells (*left panel*) that were then used to identify IL-10-secreting CD25<sup>+</sup> cells (*middle and right panels*). Representative scattergrams for MV- (*middle panel*) and mock-treated (*right panel*) samples are shown. Proportion of IL-10<sup>+</sup> cells within the CD4<sup>+</sup>CD25<sup>+</sup> population is shown as a percentage. Data obtained from FACS scattergrams were pooled and are depicted as box and whisker plots. Data shown are medians and percentiles. Statistically significant difference between the two groups and sample numbers are indicated. B, Cells were surface labeled with CD14 Ab, permeabilized, and labeled with anti-IL-10-Alexa Fluor 647 Ab. PBMC were gated and IL-10-secreting CD14<sup>+</sup> cells were identified. Representative scattergrams for MV- (*left panel*) and mock-treated (*right panel*) samples are shown. Proportion of IL-10<sup>+</sup> cells within the CD14<sup>+</sup> population is shown as a percentage. Data obtained from FACS scattergrams were pooled and are depicted as box and whisker plots. Data shown are medians and percentiles. Statistically significant difference between the two groups and sample numbers are indicated.

$p = 0.04$ ; Fig. 2, A and B). Interestingly, the level of IL-10 remained significantly higher in MV-infected samples compared with mock-infected controls for the duration of the experiment. Expression of IL-2 was also investigated, but the level in mock-treated samples was undetectable in our assay, precluding quantification, and is not shown. The secretion of cytokines in supernatant was delayed relative to gene expression (Fig. 2C) and was maximal at day 3 (IL-2), day 5 (TNF- $\alpha$ , IFN- $\gamma$ ), or day 7 (IL-10). IL-2, IL-10, TNF- $\alpha$ , and IFN- $\gamma$  were significantly increased in MV-treated relative to mock-treated cells; there was no difference in IL-4 and IL-5 levels (not shown). Interestingly, expression and secretion of IL-10 was significantly higher in MV-treated samples at all times tested except day 1. The pattern of cytokine expression and secretion was not donor dependent, because a similar pattern was observed in PBMC from five different healthy controls (data not shown).

We analyzed the mRNA and protein levels of IL-2, IFN- $\gamma$  (Th1), IL-4, IL-5, IL-10 (Th2), and TNF- $\alpha$  in patient samples (Fig. 2, D and E). Patients had elevated IL-2 ( $p < 0.001$ ), IL-4 ( $p < 0.0001$ ), IL-10 ( $p = 0.019$ ), and IFN- $\gamma$  ( $p = 0.001$ ) mRNA expression, reduced IL-5 expression ( $p < 0.001$ , not shown), and unchanged TNF- $\alpha$  expression compared with healthy controls (Fig. 2D). IL-2, IL-10, and IFN- $\gamma$  were significantly increased in patient plasma compared with controls (Fig. 2E), but plasma levels of IL-4, IL-5 (not shown), and TNF- $\alpha$  were similar in the two groups (IL-4:  $p = 0.298$ ; IL-5:  $p = 0.830$ ; TNF- $\alpha$ :  $p = 0.057$ ).

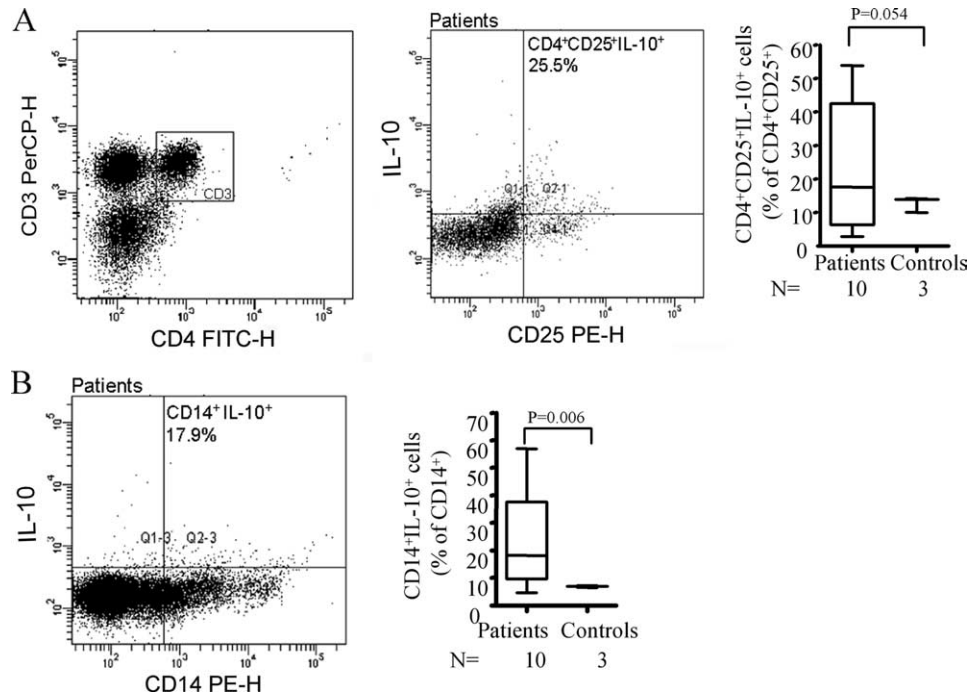
To determine the kinetics of production of IL-2, IL-10, and IFN- $\gamma$  during infection, we grouped patient samples on the basis of the day of collection after rash onset; there was an increase in all three cytokines early in infection (<3 days after rash onset) followed by a second IL-2 peak a week later; IL-10 and IFN- $\gamma$  increase was sustained over the 2-wk period (Table III).

Data shown in Fig. 2 and Table III suggest that the overall cytokine profile of adult measles patients is similar to that observed in children: IL-2, IFN- $\gamma$ , and IL-10 being high, with the latter two sustained over the period of at least 2 wk. Together, Figs. 1 and 2 show that MV infection leads to inhibition of IFN- $\alpha$ , and a mixed Th1/Th2 response with IL-10 and IFN- $\gamma$  being significantly enhanced.

#### CD4<sup>+</sup>, CD8<sup>+</sup> T cell numbers are unchanged and CD14<sup>+</sup> cells decreased in PBMC of measles patients

Previous studies (22, 34) have shown that MV infection leads to a decline in CD4<sup>+</sup> T lymphocytes in pediatric patients, starting before the onset of rash and lasting up to a month; this decline in cell number was less severe in adult patients. Our FACS data suggest that the absolute CD4<sup>+</sup> T and CD8<sup>+</sup> T lymphocyte numbers are similar between healthy controls and measles patients (Fig. 3, A–C). Interestingly, the proportion of CD4<sup>+</sup> cells within the CD3<sup>+</sup> cell population was significantly reduced as compared with healthy controls (means: 62% in patients, 51% in controls,  $p = 0.002$ ),





**FIGURE 8.** CD4<sup>+</sup>CD25<sup>+</sup> and CD14<sup>+</sup> cells in measles patients' PBMC secrete IL-10. PBMC from measles patients were cultured in RPMI 1640 containing 10% FBS for 2 h, followed by treatment with PMA, ionomycin, and brefeldin A for 3 h. *A*, Cells were labeled for CD3, CD4, CD25, and IL-10 as described in the legend to Fig. 7*A*. CD3<sup>+</sup>CD4<sup>+</sup> cells were gated to identify CD4<sup>+</sup> T cells (*left panel*), followed by identification of IL-10-secreting CD25<sup>+</sup> (*right panel*). Proportion of IL-10<sup>+</sup> cells within the CD4<sup>+</sup>CD25<sup>+</sup> population is shown as a percentage. Data obtained from scattergrams, such as the one shown, were pooled and are depicted as box and whisker plots with data from stimulated control PBMC shown for comparison. Data shown are medians and percentiles; statistical differences between the two groups and sample numbers are indicated. *B*, Cells were labeled for CD14 and IL-10 as described in the legend to Fig. 7*B*, proportion of IL-10<sup>+</sup> cells within the CD14<sup>+</sup> population is shown as a percentage. Data obtained from scattergrams, such as the one shown were pooled and are depicted as box and whisker plots with data from stimulated control PBMC shown for comparison. Data shown are medians and percentiles; statistical differences between the two groups and sample numbers are indicated.

perhaps due to an increase in total CD3<sup>+</sup> T cell population, which includes CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> T cells.

Macrophages are important in MV infection as major vectors for dissemination of MV infection (35) and as a potential source of IL-10 (19). FACS analysis showed that the number of CD14<sup>+</sup> cells in patient PBMC was significantly lower than that in healthy controls, (Fig. 4, *A* and *B*).

#### Treg are elevated in measles patient PBMC

FACS analysis showed that there was a significant increase in CD4<sup>+</sup>CD25<sup>+</sup> T cell number in PBMC of measles patients compared with healthy controls (Fig. 5*A*, *left panels* and *B*, *left histogram*). To differentiate Treg from activated T cells that also express CD25 on their cell surface, we determined the number of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup> cells in PBMC from measles patients and healthy donors (36). There was a significant increase in CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup> T cells in PBMC of measles patients compared with healthy controls (Fig. 5*A*, *right panels* and *B*, *right histogram*). There was no significant difference in the proportion of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup> within the total CD4<sup>+</sup>CD25<sup>+</sup> population between patients and controls ( $p = 0.481$ ).

#### CD14<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup> cells are associated with elevated plasma IL-10

As a first step to identify the phenotype of the cells that may be the cellular source of IL-10, CD4<sup>+</sup>CD25<sup>+</sup> T cells, CD4<sup>+</sup> T cells, and CD14<sup>+</sup> cell numbers were analyzed for their association with plasma IL-10 levels using Pearson correlation, and the data were fitted to a curve using regression analysis. There was a positive correlation between patients' plasma IL-10 level and CD14<sup>+</sup> cell

number (Fig. 6, *upper graph*) and between IL-10 and CD4<sup>+</sup>CD25<sup>+</sup> T cells (Fig. 6, *middle graph*), but no correlation between CD4<sup>+</sup> T cells and plasma IL-10 levels (Fig. 6, *lower graph*).

#### MV up-regulates IL-10-producing CD14<sup>+</sup> monocytes/macrophages and CD4<sup>+</sup>CD25<sup>+</sup> T cells

Monocyte/macrophages have been implicated as the cellular source of IL-10 in measles patients (37), and recent studies have shown that CD4<sup>+</sup>CD25<sup>+</sup> Treg secrete IL-10 and can be a major mediator of immune suppression in infection (38, 39). To determine whether CD14<sup>+</sup> monocyte/macrophages and/or CD4<sup>+</sup>CD25<sup>+</sup> T cells could be responsible for the elevated plasma IL-10, and whether MV treatment results in an increase in their cell number, we treated PBMC from healthy donors with MV (MOI = 1) or Vero-SLAM cell lysate (mock) followed 3 days later by PMA, ionomycin, and brefeldin A stimulation for 3 h before FACS analysis (Fig. 7). Following stimulation, IL-10-secreting CD4<sup>+</sup>CD25<sup>+</sup> T cells increased significantly in MV-treated compared with mock-treated PBMC (Fig. 7*A*). The proportion of IL-10-secreting CD14<sup>+</sup> cells was also significantly higher in MV-treated than in mock-treated PBMC (Fig. 7*B*).

#### CD4<sup>+</sup>CD25<sup>+</sup> T cells and CD14<sup>+</sup> monocyte/macrophages contribute to patient plasma IL-10

To confirm results obtained from the *in vitro* study, we conducted intracellular staining of IL-10 in CD4<sup>+</sup>CD25<sup>+</sup> T cells and CD14<sup>+</sup> cells in patient PBMC (Fig. 8). IL-10-secreting CD4<sup>+</sup>CD25<sup>+</sup> T cells were increased in patient compared with control PBMC ( $p = 0.054$ ; Fig. 8*A*), whereas significantly higher numbers of CD14<sup>+</sup> cells in patient PBMC secreted IL-10 compared with control

PBMC (Fig. 8B). A higher proportion of patient CD4<sup>+</sup>CD25<sup>+</sup> T cells (29%, IQR, 23–32%) than CD14<sup>+</sup> cells (6%, IQR, 4–10%) secreted IL-10 ( $p < 0.0001$ ).

## Discussion

We have examined the immune status of adult measles patients in Shanghai and found that in the acute stage of disease, patients have suppressed IFN- $\alpha$  and a mixed Th1/Th2 response with significant increases in IFN- $\gamma$  and IL-10 production. We show for the first time that adult measles patients, like children, have sustained high IL-10 levels; IL-10 is secreted by both monocytes/macrophages and CD4<sup>+</sup>CD25<sup>+</sup> T cells. We also show for the first time that CD4<sup>+</sup>CD25<sup>+</sup> Treg are increased in PBMC of adult measles patients. To the best of our knowledge, this is the first detailed study to investigate the cytokine expression and secretion induced by natural measles infection in adults.

MV passage history has been shown to influence its ability to induce type I IFN (40); Vero cell-adapted MV is a strong IFN- $\alpha$  inducer (41), whereas wild-type MV almost completely suppresses IFN- $\alpha$  production (25). The clinical isolate of MV used in this study was a strong IFN- $\alpha$  suppressor both in vivo and in cell culture.

Cytokines play critical roles in regulating the outcome of Ag-specific T cell responses. In our study, MV infection primed a transient IL-2 secretion, as well as a robust plasma IFN- $\gamma$  and IL-10 production that lasted for 2 wk after rash onset. An increase in IFN- $\gamma$  in measles-infected children compared with healthy controls has been observed in other studies and may serve to inhibit viral growth and limit the spread of infection (42). However, in contrast to our findings of sustained increase, these studies have found a transient increase in both IL-2 and IFN- $\gamma$ , lasting for a few days following rash (19, 22), followed by sustained IL-4 production (19). We did not find any increase in IL-4 secretion in adult patient plasma. A similar response was observed when a clinical isolate of MV was used to infect PBMC; an early IL-4 gene induction that was not reflected in protein secretion may be due to uptake of secreted IL-4 by cells, and does not necessarily reflect lack of protein production. Similar findings have been reported in a study where PBMC from previously immunized adults were infected with MV. All subjects produced IFN- $\gamma$ , and in subjects who produced both IFN- $\gamma$  and IL-4, maximal IFN- $\gamma$  production in vitro always greatly exceeded that of IL-4 (43).

Th1 responses with high levels of IFN- $\gamma$  have variously been shown to be protective against pathogens (44) or to lead to enhanced inflammation (45). Previous work has shown that PBMC from children produce lower levels of IFN- $\gamma$  when infected with MV than adult PBMC (46), perhaps a result of limited cellular immunity in infants. The sustained IFN- $\gamma$  secretion in plasma of adult measles patients in this study may have implications for virus clearance, enhanced tissue inflammation, and the observed disease severity.

There did not appear to be any difference in IL-5 secretion between patients and healthy controls and also MV-infected and non-infected PBMC; we did observe a decrease in IL-5 gene expression in patients. In Zambian children plasma IL-5 levels were lower in patients compared with controls (19). In contrast, a significant up-regulation of IL-5 mRNA has been reported among seropositive adult donors after vaccination (47). The role of IL-5 in MV infection is not clear and data may be complicated by the underlying allergic status of the subjects.

Interestingly, we did not observe a general decrease in CD4<sup>+</sup> and CD8<sup>+</sup> T cells in adult measles patients, although there was a trend toward lower levels compared with healthy donors. Our data are in agreement with a previous report from Japan showing re-

duction of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in acute measles; the reduction was significantly more severe in children than in adults as compared with age-related matched controls (34). We observed an increase, albeit insignificant, in the total CD3<sup>+</sup> T cells in patient PBMC compared with controls, possibly due to expansion of a CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> T cell subset that defines a double negative Treg phenotype (48). Double negative Treg inhibit immune responses by directly killing effector T cells in an Ag-specific fashion, and produce IFN- $\gamma$  and TNF- $\alpha$  in addition to other cytokines. Further detailed investigations are needed to determine what role these cells may play in MV pathology.

There was a significant reduction in total number of CD14<sup>+</sup> monocytes/macrophages in patients compared with controls. MV is known to infect monocytes/macrophages and may directly induce production of IFN- $\gamma$  or IL-10. Stimulation of monocytes by differentiating agents such as IFN- $\gamma$  leads to marked down-regulation of CD14 expression (49), and cells might migrate to the sites of MV-induced rash, which may lead to the observed reduction in cell number in PBMC.

Interestingly, there was a significant increase in CD4<sup>+</sup>CD25<sup>+</sup> T cells. Recently, it was shown that low surface expression of CD127 is inversely correlated with FoxP3 expression, and in combination with surface expression of CD25 it can distinguish between human regulatory and conventional CD4<sup>+</sup> T cells in human blood (36, 50). We have used this information to analyze the Treg population within total CD4<sup>+</sup>CD25<sup>+</sup> T cells and show that Treg were significantly increased in the peripheral blood of adult measles patients compared with healthy controls.

This is the first report of increased Treg in measles patients; Treg may have a role in the observed immunosuppression. Treg act to prevent infection-induced immunopathology or to sustain pathogen persistence by suppressing protective Th1 responses (51). Both natural and inducible IL-10-producing Treg affect the magnitude of antiviral immunity, contributing in some instances to viral persistence and chronic disease (26).

Sustained high levels of IL-10 during convalescence suggest a role for this immunoregulatory cytokine in MV-induced immunosuppression. Similar to the observations in this study, plasma levels of IL-10 remained elevated for weeks in children with MV infection (19). The increased IL-10 levels may also be implicated in the decrease in IL-5 expression, because IL-10 is known to inhibit IL-5 production by T cells and in mouse models of allergic disease (52, 53). IL-10 has been shown to display a range of immune suppressive effects, including inhibition of APC function, induction of anergy, differentiation of Treg, and control of the expansion of other T cell populations (54), and may be key to the observed decrease in monocyte/macrophages and innate immune responses observed in MV infection.

The cellular source of the increased IL-10 in MV infection is not clear and various cell populations, including macrophages, have been suggested (19). IL-10 can be produced by many different myeloid and lymphoid cells, and more than one population of IL-10-producing cells may be induced during an infection (reviewed in Ref. 55). Dendritic cells, macrophages, Th1 cells, and Treg have all been shown to produce IL-10 in different disease models.

Our data show that in adult measles patients, monocyte/macrophages and CD4<sup>+</sup>CD25<sup>+</sup> T cells (including effector T cells and Treg) contribute to increased IL-10 production. Although a role for IL-10 in suppression by Treg has long been excluded, in several in vivo systems suppression by Treg is mediated by inhibitory cytokines like IL-10 (38, 56). A recent study has shown that a subset of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg, defined by the surface expression of TIRC7, is FoxP3<sup>+</sup>IL-10<sup>+</sup> (39), further consolidating the case for IL-10-mediated suppression by CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg.

In conclusion, our data show for the first time that CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low</sup> Treg are up-regulated in MV infection, and that CD4<sup>+</sup>CD25<sup>+</sup> T cells and CD14<sup>+</sup> monocyte/macrophages contribute to the increased IL-10. Whether these two cell types are solely responsible for the IL-10 increase or whether other cell types are also involved awaits further detailed investigations. We also show that, as observed previously in children (57), adult measles patients have significantly reduced IFN- $\alpha$  production and significantly increased sustained IFN- $\gamma$  and IL-10 secretion. The enhanced Treg, IFN- $\gamma$ , and IL-10 being implicated in immunopathology and immunosuppression may together be the underlying cause of the observed clinical severity in adult MV patients.

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## Disclosures

The authors have no financial conflict of interest.

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