CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Mar. 2002, p. 295–298 Vol. 9, No. 2
1071-412X/02/$04.00+0 DOI: 10.1128/CDLI.9.2.295–298.2002

Influence of Specimen Age and Use of Different Negative Controls in Determination of Intracytoplasmic Levels of Cytokines after Whole-Blood Culture Assay

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Received 27 March 2001/Returned for modification 20 June 2001/Accepted 6 November 2001

Intracytoplasmic detection of cytokines by flow cytometry has become a powerful tool in the characterization of cytokine-producing cells. However, it is not known to what extent specimen age and the use of various negative controls may influence the amount of cytokine-positive cells. We therefore compared different times of storage and the use of several negative controls in the determination of intracytoplasmic levels of cytokines. There was a substantial decline of interleukin-2- and gamma interferon-positive lymphocytes after 20 h and especially after 48 h of storage. The precision of intracytoplasmic interleukin-6 determination decreases after long-term storage compared to 2 h of storage, whereas the amount of interleukin-8-positive monocytes remained rather stable. Therefore, we recommend performing the analysis as fast as possible after the blood sample is drawn. Under consideration of isotype-matched antibodies and nonstimulated cells as negative controls instead of the purified antibody-blocking control, strikingly higher amounts of interleukin-2-, gamma interferon, interleukin-6-, and interleukin-8-positive cells were found. For a meaningful interpretation of data these differences have to be kept in mind. Further studies should evaluate the exact specificity of these controls.

Intracytoplasmic detection of cytokines by flow cytometry has revolutionized the area of cell biology in the past few years (7, 9, 10). It represents a powerful analytical technique in which individual cells can be simultaneously analyzed for several parameters, including cell size and granularity, as well as the coexpressed levels of surface and intracellular markers defined by fluorescent antibodies. If this technique is performed after whole-blood culture assay, cellular interactions are preserved and cell activation by the use of separation procedures can be avoided (4, 6, 11). However, it is a disadvantage of the assay that whole blood cannot be stored for a longer period. The aim of this study was to investigate the impact of specimen age on the determination of intracytoplasmic levels of cytokines. Furthermore, the enhancement of nonspecific binding during the fixation and stimulation procedure (8) makes a correct interpretation of data without the use of adequate negative controls difficult (11). It has been demonstrated that a surplus of purified anticytokine antibodies blocks specific binding and allows an excellent differentiation between positively and negatively stained cells (8). However, isotype-matched antibodies and nonstimulated cells were also used as negative controls in several studies (1–3, 5, 12–15). The objective of this study was to compare these negative controls in the determination of intracytoplasmic levels of cytokines.

MATERIALS AND METHODS

Study population. Blood was obtained from healthy adult volunteers after informed consent.

Reagents. Lipopolysaccharide (LPS), phorbol 12-myristate 13-acetate (PMA), ionomycin, and monensin were obtained from Sigma (Deisenhofen, Germany); paraformaldehyde and saponin were obtained from Riedel de Haen (Seelze, Germany); RPMI medium, Hanks' balanced salt solution (HBSS), HEPES buffer, glutamine, pyruvate, nonessential amino acids, and penicillin-streptomycin were obtained from Seromed Biochrome (Berlin, Germany); and Immuno-Lyse solution was obtained from Coulter Electronics (Krefeld, Germany).

MAbs. The following monoclonal antibodies (MAbs) were purchased from Pharmingen (Heidelberg, Germany): anti-human CD3-CyChrome (17A2, rat immunoglobulin G2b [IgG2b]), CD14-PE (M5E2, mouse IgG2a), interleukin-2–fluorescein isothiocyanate (IL-2–FITC) (MQ1-17H12, rat IgG2a), gamma interferon-phycoerythrin (IFN-γ–PE) (4S.B3, mouse IgG1), IL-6–FITC (MQ2-13A5, rat IgG1), IL-8–FITC (G265-8, mouse IgG2b); purified anti-human IL-2 (MQ1-17H12), purified anti-human IFN-γ (4S.B3), purified anti-human IL-6 (MQ2-13A5), purified anti-human IL-8 (G265-8); and isotype-matched antibodies against rat IgG2a (R35-95, FITC), mouse IgG1 (MOPC-21, PE), rat IgG1 (R3-34, FITC), and mouse IgG2b (27-35, FITC).

Culture and stimulation of cells. Heparinized whole blood was either processed immediately or stored at room temperature for 2, 20, or 48 h before processing, as indicated. After that it was suspended in RPMI 1640 supplemented with 1% penicillin-streptomycin, 2 mM glutamine, 1 mM pyruvate, and nonessential amino acids at a concentration of 5×10^6 leukocytes/ml. Aliquots (1.5 ml) were incubated 5 h at 37°C with 5% CO_2 in multiwell plates with PMA (3 μg/ml) and 3 μM ionomycin to induce IFN-γ and IL-2 synthesis in lymphocytes and with LPS (30 ng/ml) to induce IL-6 and IL-8 production in monocytes. Simultaneously, cells were exposed to monensin at a final concentration of 3 μM to block cytokine secretion. After a washing step with HBSS, cultured cells were fixed in 4% paraformaldehyde for 10 min and resuspended in nonfat dry milk (5%) for 16 h at 4°C in the dark.

Intracellular staining of cytokines. Cells were washed in HBSS and resuspended in a buffer consisting of HBSS, 0.1% saponin, and 0.01 M HEPES buffer. Aliquots (200 μl) of cells were added to tubes containing 0.5 μg (10 μl) of MAbs against CD3, CD14, IFN-γ, IL-2, IL-6, and IL-8, respectively. The following controls were used as negative controls.

(i) Purified antibody-blocking control. Stimulated cells were incubated with 5 μg (10 μl) of unlabeled anticytokine MAbs for 20 min at 4°C in the dark before the addition of fluorochrome-labeled MAbs of an identical specificity.

(ii) Isotype control. Stimulated cells were incubated with 5 μg (10 μl) of fluorochrome-conjugated antibody of irrelevant specificity, which has an identical immunoglobulin isotype to the anticytokine antibody being stained, for 20 min at 4°C in the dark.

(iii) Nonstimulated cells. Cells incubated without PMA, ionomycin, or LPS were stained with 0.5 μg (10 μl) of fluorochrome-labeled anticytokine MAbs for

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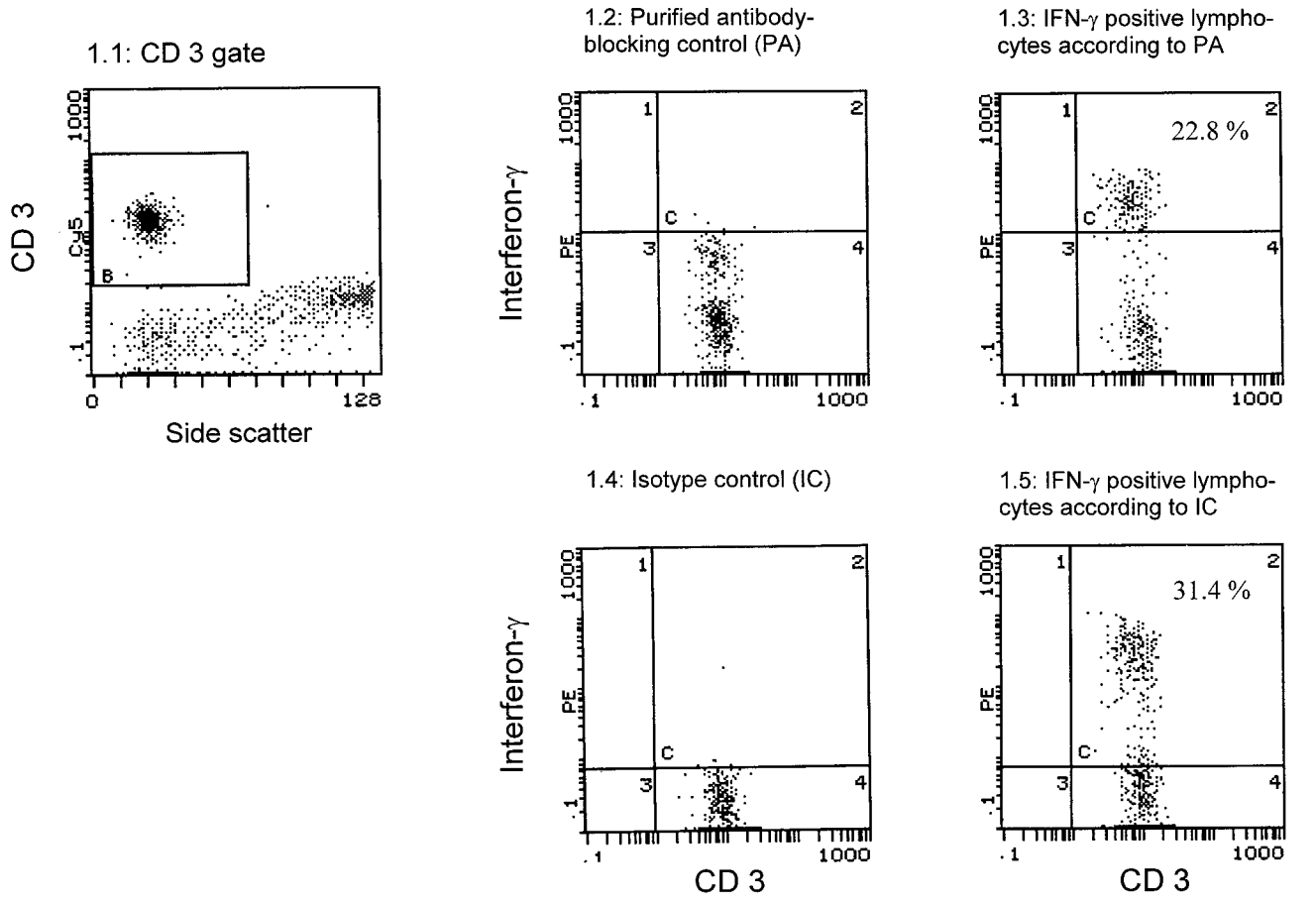

FIG. 1. Discrimination of specific and nonspecific staining by the use of the purified antibody-blocking control (PA) and isotype control (IC) in a representative case. Panel 1.1 demonstrates gating on CD3-positive lymphocytes stained with cychrome-conjugated CD3 (*y* axis) on a logarithmic scale versus side-scatter characteristics on a linear scale (*x* axis). All CD3-positive cells are further evaluated in panels 1.2 to 1.4, which display staining with PE-conjugated IFN-γ (*y* axes) versus cychrome-conjugated CD3 (*x* axes) on a logarithmic scale. The quadrants (C) were set according to the purified antibody-blocking control (panels 1.2 and 1.3) or isotype control (panels 1.4 and 1.5). IFN-γ-positive monocytes are represented in dot plots in the right upper quadrants (2). The use of the purified antibody-blocking control prior to cytokine staining abrogates selectively specific staining and exposes the peak of nonspecific staining (panels 1.2 and 1.3). In contrast, by using isotype-matched antibodies considerably fewer events are seen, as is illustrated by the lower level of quadrant C, which defines the boundary between positive and negative events (panel 1.4). Consequently, higher amounts of IFN-γ positive lymphocytes are reported according to the isotype control (31.4%) (panel 1.5) compared with the purified antibody-blocking control in this representative case (22.8%) (panel 1.3).

20 min at 4°C in the dark. No isotype control or purified blocking antibodies were added.

Flow cytometric analysis. Three-color flow cytometric analysis was performed on an EPICS XL flow cytometer with System II, version 1.0, software that had been calibrated daily with DNA-Check-Beads (Coulter Electronics). A total of 5,000 CD3-positive lymphocytes and 2,000 CD14-positive monocytes were acquired from each sample gating on CD3- and CD14-positive cells (Fig. 1, panel 1.1). Dead cells were excluded by forward- and side-scatter gating. Cytokine positivity was defined by setting regions with the lower limits for cytokine positivity below 2% determined from the above-mentioned controls (Fig. 1, panels 1.2 and 1.4). Data were expressed as the percentage of cytokine-producing lymphocytes and monocytes.

Statistical analysis. The difference between 2 h of specimen age and 20 or 48 h of specimen age was reported in two independent experiments with six healthy volunteers, respectively. The mean deviation between the investigated negative controls was calculated from 20 independent experiments. Data were expressed as mean deviation in percent and 95% confidence interval (95% CI). Statistical differences were tested by the Wilcoxon signed-rank test. A P of less than 0.05 was considered statistically significant. Statistical analyses were performed using SPSS 9.0 statistical software (SPSS Inc., Chicago, Ill.). The intra-assay variability of the method was 3.9% (95% CI, 2.2 to 5.6%) for all cytokines investigated. It was calculated from two analyses of the same sample for each cytokine for three different healthy volunteers, respectively.

RESULTS

Impact of specimen age on determination of intracytoplasmic levels of cytokines. A mean decline of 11.7% occurred after 20 h (95% CI, −2.5 to 25.8%; $P = 0.02$), and a 52.2% decline of IL-2-positive lymphocytes occurred after 48 h of storage (95% CI, 43 to 61.4%; $P < 0.0001$) (Fig. 2, panel 2.1). The amount of IFN-γ-positive lymphocytes remained rather stable after 20 h of storage (6.9%; 95% CI, −2.4 to 16.3%; $P = 0.17$). However, after 48 h a 31.9% decline of IFN-γ-positive lymphocytes was seen (95% CI, 16 to 47.8%; $P = 0.02$), as well (Fig. 2, panel 2.1). The amount of IL-6-positive monocytes became more inaccurate with both rising and falling values after 20 h (6.2%; 95% CI, −17.6 to 30%; $P = 0.55$) and 48 h

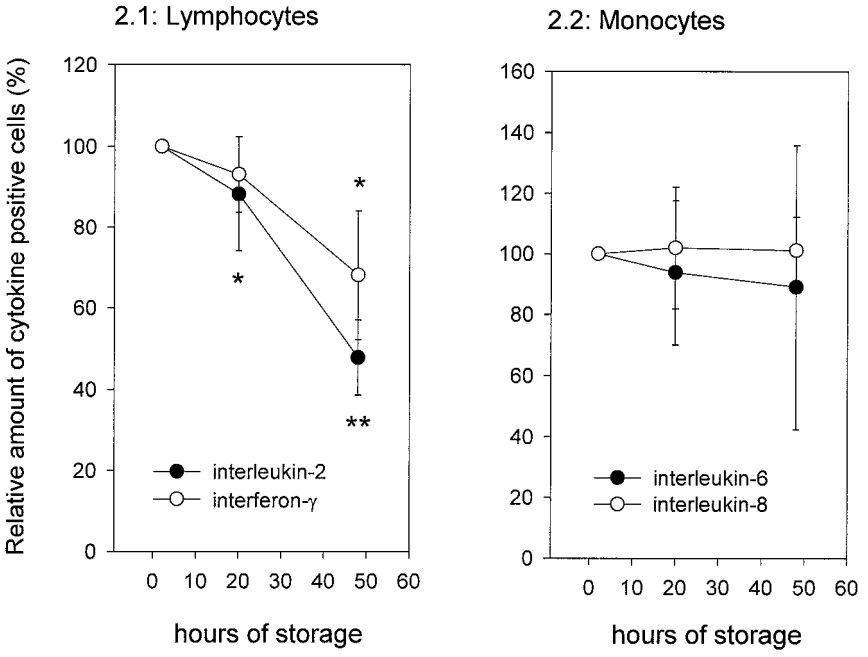

FIG. 2. Influence of specimen age on the amount of cytokine-positive lymphocytes and monocytes. The amount of IL-2- and IFN-γ-positive lymphocytes decreases considerably after long-term storage (panel 2.1). There was an increasing imprecision with both rising and falling values for IL-6-positive monocytes after 48 h of storage. In contrast, the amount of IL-8-positive monocytes remained rather stable during long-term storage (panel 2.2). The data are plotted as mean percentages of the initial amount of cytokine-positive cells, which was set as 100% for six healthy volunteers. Differences were significant at a level of $P < 0.05$ (*) or $P = 0.0001$ (**), as indicated. Bars refer to 95% confidence interval.

(10.9%; 95% CI, −35.8 to 57.6%; $P = 0.41$) compared with 2 h of storage (Fig. 2, panel 2.2). The amount of IL-8-positive monocytes remained moderately stable after 20 h (−2%; 95% CI, −22.1 to 18.1%; $P = 0.86$) and 48 h of storage (−1.2%; 95% CI, −12.3 to 10%; $P = 0.79$) (Fig. 2, panel 2.2).

Comparison between isotype-matched antibodies and the purified antibody-blocking control. Whole blood was processed immediately after the sample was drawn. The efficiency of the purified antibody-blocking control to prevent staining with fluorochrome-labeled antibody is demonstrated in a titration experiment shown in Table 1. The results obtained with the isotype control differed tremendously from those obtained with the purified antibody-blocking control. The greatest mean difference was seen for IL-8, with a 44.9% higher amount of IL-8 positive monocytes (95% CI, 15.9 to 112%; $P < 0.0001$) compared to the purified antibody-blocking control, followed by a 29% higher amount of IL-6-positive cells (95% CI, 8.4 to 75%; $P < 0.0001$), a 19.4% higher amount of IFN-γ-positive cells (95% CI, 3.6 to 49%; $P < 0.0001$), and a 14.5% higher amount of IL-2-positive cells (95% CI, 2 to 40%; $P < 0.0001$). Except for 2 out of 20 samples for IL-6 the amount of cytokine-positive cells was above that of the purified antibody-blocking control in all investigated cases. The capability of both negative controls to discriminate specific from nonspecific staining is illustrated in Fig. 1, panels 1.2 to 1.5, in a representative case for intracytoplasmic IFN-γ staining.

Comparison between nonstimulated cells and purified antibody-blocking control. If the threshold is set according to nonstimulated cells as a negative control, then the number of IL-6-positive monocytes was 69.3% higher (95% CI, 1.5 to 267%; $P < 0.0001$) than that obtained with the purified antibody-blocking control, followed by a 26.9% higher amount of IFN-γ-positive lymphocytes (95% CI, 9.6 to 60.1%; $P < 0.0001$) and a 18.8% higher amount of IL-2-positive lymphocytes (95% CI, 6.4 to 47.6%; $P < 0.0001$). In the determination of IL-8, 6 values were below and 14 values above those of the purified antibody-blocking control (18.7%; 95% CI, 4.4 to 36.7%; $P < 0.05$). Under consideration of purified blocking

TABLE 1. Efficiency of purified antibodies to block specific binding

Cytokine	Mean % (range) cytokine-positive cells				
	None	0.5 μg	2 μg	5 μg	10 μg
IL-2	100	37.2 (33–45)	7.3 (4.5–9.4)	1 (0.7–1.5)	0.3 (0.1–0.5)
IFN-γ	100	99.8 (94–110)	50.7 (29–71)	6 (4.4–8.5)	1.4 (0.1–2.4)
IL-6	100	16.9 (12–26)	5.3 (4.3–7.3)	2.4 (1.9–3.3)	1.8 (1–2.4)
IL-8	100	66 (55–74)	29.2 (23–35)	1.1 (0.7–1.8)	0.2 (0.1–0.3)

[a] Stimulated cells were preincubated with increasing concentrations of purified anticytokine antibodies (0.5, 2, 5, and 10 μg) before the addition of 0.5 μg of fluorochrome-labeled antibodies of an identical specificity. Values were obtained from three different experiments. The percentage of cytokine-positive cells is reported compared to stimulated cells which were incubated without purified anticytokine antibodies (set as 100%).

antibodies as a reference negative control we detected IL-6- and IL-8-positive monocytes in nonstimulated cells in 3 out of 50 and 8 out of 50 adult subjects, respectively (data not shown).

DISCUSSION

Determination of cytokines by flow cytometry at the single-cell level has become a powerful tool in the characterization of cytokine-producing cells (7, 9, 10). However, several factors must be considered for a meaningful interpretation of data. Here, we demonstrate that storage of whole blood before processing did considerably influence the amount of cytokine-positive cells. This effect was greatest for IL-2 and IFN-γ after 48 h of storage, with a 52.2 and 31.9% mean decline of cytokine positive lymphocytes, respectively. Moreover, there was an increasing imprecision with both rising and falling values for IL-6-positive monocytes after 48 h compared with 2 h of storage. In contrast, the number of IL-8-positive monocytes remained rather stable after both 20 and 48 h of storage (Fig. 2, panels 2.1 and 2.2). Under consideration of these data, we recommend performing the analysis as fast as possible, especially for IL-2, IFN-γ, and IL-6, but not later than 20 h of storage.

Furthermore, we demonstrated a profound difference between frequently used negative controls. This is of special concern because the procedure of stimulation, fixation, and permeabilization induces an enhancement of nonspecific binding (8). The use of an adequate negative control is crucial in this setting to discriminate between cytokine-specific and nonspecific background staining. It has been demonstrated by others that a surplus of purified anticytokine antibodies blocks specific binding and allows an excellent differentiation between positively and negatively stained cells (8). Under consideration of the frequently used isotype-matched antibodies, strikingly higher amounts of all cytokines were found compared with the purified antibody-blocking control. The difference was remarkable, especially for IL-6 and IL-8, with a 29 and 44.9% higher amount of cytokine positive monocytes, respectively. However, 14.5 and 19.4% higher amounts of IL-2- and IFN-γ-positive lymphocytes were documented, as well. The discrepancy between isotype-matched antibodies and the purified antibody-blocking control may be explained by a profound increase in nonspecific binding of antibodies to cells after the procedure of stimulation, permeabilization, and fixation. Isotype controls are antibodies of irrelevant specificity with an immunoglobulin isotype identical to that of the anticytokine antibody being stained. They only display the inherent staining background for a given fluorescent antibody (8). However, it is questionable to what extent isotype-matched antibodies are able to detect the enhancement of nonspecific staining induced by the procedure in the same way as the purified antibody-blocking control demonstrated by Prussin et al. (8).

The use of nonstimulated cells instead of the purified antibody-blocking control demonstrated strikingly higher amounts of IL-6 (69.3%)-, IFN-γ (26.9%)-, and IL-2 (18.8%)-positive cells. For IL-8 both higher and lower amounts of positive monocytes were seen. If purified antibodies were used as a staining control, IL-6- and especially IL-8-positive monocytes could be detected without stimulation. If the threshold is set according to these cells, the number of false-negative cells will necessarily rise. This shortcoming does explain the lower amount of IL-8-positive monocytes in a few samples comparing the use of nonstimulated cells to the purified antibody-blocking control. Because nonstimulated cells lack stimulation they cannot display the complete enhancement of nonspecific staining, which is in part induced by the stimulation procedure (8). Both pitfalls qualify nonstimulated cells as an unreliable staining control apart from their ability to detect spontaneous cytokine production.

In conclusion, we have clearly demonstrated that specimen age has a tremendous effect on the number of cytokine-positive cells detected when the specimen is analyzed by flow cytometry. For a meaningful interpretation of data, we recommend the assay be performed as early as possible after the blood sample has been drawn. Moreover, this is the first report demonstrating a profound difference between commonly used negative controls. This has to be kept in mind in the interpretation of data. Further studies should evaluate what kind of negative control has the highest precision in the differentiation between positively and negatively stained cells.

ACKNOWLEDGMENT

This work was in part supported by Lübeck Hilfe für krebskranke Kinder, e.V.

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ERRATA

Elucidation of the Cross-Reactive Immunoglobulin M Response to Human Herpesviruses 6 and 7 on the Basis of Neutralizing Antibodies

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Volume 9, no. 2, p. 394–402, 2002. Page 394: The returned for modification date should read "2 October 2001."

Influence of Specimen Age and Use of Different Negative Controls in Determination of Intracytoplasmic Levels of Cytokines after Whole-Blood Culture Assay

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Volume 9, no. 2, p. 295–298, 2002. Page 297, column 1: line 7 from bottom and below should read as follows. "The greatest mean difference was seen for IL-8, with a 44.9% higher amount of IL-8 positive monocytes (95% CI, 33.7% to 56%; $P < 0.0001$) compared to the purified antibody-blocking control, followed by a 29% higher amount of IL-6-positive cells (95% CI, 16.7% to 41.3%; $P < 0.0001$), a 19.4% higher amount of IFN-γ-positive cells (95% CI, 14.3% to 24.6%; $P < 0.0001$), and a 14.5% higher amount of IL-2 positive cells (95% CI, 10.7% to 18.2%; $P < 0.0001$)."

Page 297, column 2: line 10 from bottom and below should read as follows. " . . . then the number of IL-6 positive monocytes was 69.3% higher (95% CI, 37.8% to 100.7%; $P < 0.0001$) than that obtained with the purified antibody-blocking control, followed by a 26.9% higher amount of IFN-λ-positive lymphocytes (95% CI, 20.9% to 32.8%; $P < 0.0001$) and a 18.8% higher amount of IL-2-positive lymphocytes (95% CI, 13.7% to 24%; $P < 0.0001$). In the determination of IL-8, 6 values were below and 14 values above those of the purified antibody-blocking control (18.7%; 95% CI, 14.5% to 22.9%; $P < 0.05$)."