Enzyme-Linked Immunospot Assays Provide a Sensitive Tool for Detection of Cytokine Secretion by Monocytes

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Blood monocytes as well as tissue-differentiated macrophages play a pivotal role in controlling immune reactions. Monocytes regulate the extent, nature, and duration of immune responses by secretion of cytokines. Interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), IL-10, and IL-12 are of particular interest, since IL-12 shifts the immune response towards a Th1 type, facilitating the production of, e.g., TNF-α and IL-6, while IL-10 counteracts Th1 responses and promotes the production of Th2-related cytokines such as IL-4. A tight regulation of these four cytokines keeps the balance and decides whether Th1 or Th2 will predominate in immune reactions. Enzyme-linked immunospot (ELISPOT) assays are among the most-sensitive and -specific methods available for cytokine research. They permit ex vivo identification of individual cells actively secreting cytokines. In the present study we prepared monocytes from healthy subjects' blood and adapted ELISPOT assays to define optimal conditions to detect and enumerate monocytes secreting IL-6, TNF-α, IL-10, and IL-12. The optimal time for monocyte incubation was 24 h, and optimal monocyte numbers (in cells per well) were 2,000 for IL-6, 1,000 for TNF-α, 50,000 for IL-10, and 100,000 for enumeration of IL-12 secreting monocytes. Among healthy subjects, 10% ± 5% of the monocytes secreted IL-6, 12% ± 12% secreting TNF-α, 0.1% ± 0.1% secreted IL-10, and 0.2% ± 0.3% secreted IL-12 (values are means ± standard deviations). In conclusion, ELISPOT assays constitute a valuable tool to enumerate monocytes secreting IL-6, TNF-α, IL-10, and IL-12 and probably to enumerate monocytes secreting other cytokines and proteins.

Materials and Methods

Donors. Blood specimens were obtained from 49 healthy subjects (25 females) with a mean age ± standard deviation of 43 ± 12 years, consisting of staff from the Neuroimmunology Department and blood donors.

Antibodies and cytokines. Monoclonal antibodies (MAb) to human IL-6, IL-10, and IL-12p40 and biotinylated MAb to human IL-6, IL-10, and IL-12p40 were all from Mabtech (Stockholm, Sweden), and MAb to TNF-α and biotinyl-
ated polyclonal antibody to TNF-α were from R&D Systems (R&D Systems, Oxoid, United Kingdom).

Peridinin chlorophyll protein-labeled MAb to CD3; phycoerythrin (PE)-la-
beled MAb to CD14, CD80, CD86, HLA-ABC, and HLA-DR; fluorescein iso-
thioceyanate (FITC)-labeled MAb to CD15, CD19, and CD56 were obtained from Becton Dickinson (Mountain View, Calif.). PE-labeled MAb to CD40 was from Serotec (Oxford, United Kingdom). FITC-labeled MAb to CD14 and irrelevant isotype-matched MAb were PE-immunglobulin G1 (IgG1) and FITC-IgG1 from Dako (Copenhagen, Denmark).

Preparation of MNC. Blood mononuclear cells (MNC) were separated by density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway). Cells from the interphase were collected, washed three times with Dulbecco’s modi-
fication of Eagle’s medium (Gibco, Paisley, United Kingdom) and supplemented with antibiotics, 10% fetal calf serum (Gibco), 1% minimal essential medium (Gibco), and 1% l-glutamine (Gibco). Cells were adjusted to a concentration of 10^6 cells/ml. Cell viability as measured by trypan blue exclusion always exceeded 95%.

Preparation of monocytes. Percoll (Pharmacia, Uppsala, Sweden) separation (6, 14) with modifications was used to prepare monocytes from blood. A total of 20 × 10^6 MNC were collected and centrifuged at 500 × g for 10 min at 4°C. Supernatant was discarded and cells were carefully resuspended in 60% (vol/vol) Percoll in complete medium. Subsequently, 47.5 and 34% Percoll in complete medium was layered upon the cell suspension. Cells were centrifuged for 40 min at 1,700 × g. The low-density fraction representing monocytes, as confirmed by flow cytometry, was collected and washed twice with complete medium. Cells were adjusted to a concentration of 10^6 cells/ml, and cell viability as measured by trypan blue exclusion always exceeded 95%.

Detection of IL-6, TNF-α, IL-10, and IL-12-secreting mononuclear cells by ELISPOT assays. To detect and enumerate IL-6, TNF-α, IL-10, and IL-12-secreting MNC and monocytes, ELISPOT assays as originally described by Czerkinsky and collaborators (7) were previously adapted to blood samples. Aliquots containing 10^5 monocytes, respectively, were applied to individual wells in duplicate. These cell numbers were found in kinetic studies to yield high numbers of cells secreting the cytokines under study. As a positive control, aliquots of monocytes were stimulated with lipopolysaccharide (LPS) at a concentration of 10^6 cells/ml. Cell viability as measured by trypan blue exclusion always exceeded 95%.

Preparation of MNC for IL-6, TNF-α, IL-10, and IL-12 production by ELISA were from R&D Systems (R&D Systems, Oxoid, United Kingdom).

Peridinin chlorophyll protein-labeled MAb to CD3; phycoerythrin (PE)-la-
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Detection of IL-6, TNF-α, IL-10, and IL-12-secreting mononuclear cells and mono-
cellular cells by ELISPOT assays. To detect and enumerate IL-6, TNF-α, IL-10, and IL-12-secreting MNC and monocytes, ELISPOT assays as originally described by Czerkinsky and collaborators (7) were previously adapted to blood samples (9–11). Here we adapted ELISPOT assays to detect and enumerate monocytes secreting cytokines. Microtiter plates with nitrocellulose bottoms (Multiscreen-HA plates; Millipore, Molsheim, France) were coated for 2 h at 37°C with (per well) 100 μl of MAb to human IL-6 (13A5), IL-10 (9-D7), IL-12p40 (13A5) (all from Mabtech), or TNF-α (200111; R&D) diluted in sterile filtered phosphate-buffered saline (PBS) (pH 7.4) to a concentration of 10 μg/ml. The IL-12p40 antibody applied in this study is capable of detecting both the IL-12p70 subunit as well as the bioactive IL-12p70 (19). After removal of coating solutions by washing plates six times with 200 μl of PBS and subsequent removal of excess PBS by flicking, 200-μl aliquots containing 1 × 10^3, 2 × 10^3, 5 × 10^3, and 1 × 10^4 monocytes, respectively, were applied to individual wells in duplicate. These cell numbers were found in kinetic studies to yield high numbers of cells secreting the cytokines under study. As a positive control, aliquots of monocytes were stimulated with lipopolysaccharide (LPS) at a final concentra-
tion of 10 μg/ml. As a negative control, complete medium was also added to wells in the absence of cells, or cells were added to wells devoid of primary antibody. Plates were incubated at 37°C for 24 h in humidified air containing 5% CO2 and then twice washed with 200 μl of PBS, and subsequently emptied and washed six times with 200 μl of PBS and subsequently emptied and washed six times with 200 μl of PBS and 100 μl of 1% bovine serum albumin (BSA)-PBS. Ten thousand events were acquired in a Becton Dickinson FACScan flow cytometer. This instrument was calibrated twice per month for fluorescence intensity (Ca-
librie; Becton Dickinson). Control samples were stained with MAb of similar isotype but irrelevant specificity.

Data analysis. Flow cytometer data were processed using CellQuest software (Becton Dickinson). To check the purity of the monocytes obtained by density gradient centrifugation, each sample was stained using anti-CD14 (monocyte-subpopulation), anti-CD3 (T cells), and anti-CD19 (B cells) MAb. B- and T-cell contamination never exceeded 10%. In preliminary experiments, contamination by NK cells (CD56) and granulocytes (CD14, CD15) was checked. The per-
centages of NK cells and granulocytes never exceeded 0.25% of the gated population and were omitted during further analyses. The phenotypes of the obtained monocytes and the CD14-positive monocyte subpopulation were monitored with standard combinations of anti-CD14 versus anti-CD80, -CD86, -CD40, -HLA-DR, and -HLA-ABC. Nonisotypic isotype-matched IgOs conjugated to the same fluorochromes were used as negative controls.

All materials and chemicals as listed above were endotoxin free as tested by the manufacturers. All solutions, including the water used to prepare the PBS and the medium, never contained concentrations or endotoxin of <0.05 ng/ml as determined using E-toxate Limulus amoebocyte assay (Gibco).

The study protocol was approved by the Ethical Committee of Karolinska Institutet at Huddinge University Hospital, and informed consent was obtained from all healthy subjects.

Statistical analysis. The Wilcoxon signed rank test was used when paired samples of the same individual were compared. The nonparametric Kruskal-
Wallis analysis of variance test was used to compare more than two groups simultaneously. Upon obtaining a significant P value (P < 0.05) for Kruskal-
Wallis analysis of variance test, reflecting differences between one versus another group(s) in the study, a multiple-comparisons test (25) was used to determine which groups under study were differing. Spearman’s rank test was used for correlations.

RESULTS

Analysis of purity and phenotype of isolated monocytes. Purity of the prepared monocyte population as examined by flow cytometry with anti-CD3 and anti-CD19 MAb always exceeded 90% (mean purity, 95%). Monocytes typically expressed high levels of CD14 (76% ± 16%), HLA-ABC (90% ± 10%), HLA-DR (86% ± 16%), CD86 (82% ± 13%), CD80 (7% ± 7%), and CD40 (21% ± 18%) (values are presented as mean ± standard deviation [SD]) (Fig. 1). Occurrence of these markers and morphological characteristics of monocytes (see Fig. 4A) is in accordance with the role of monocytes/macrophages as antigen presenting cells.

Analyses of monocyte recovery and yield. To evaluate possible loss of monocytes during Percoll density gradient centrif-
ugation, two experiments, each including five healthy controls, were performed. MNC and high- and low-density fractions after Percoll separation were collected and analyzed in parallel. MNC contained on an average 8% monocytes, while the low-density fraction contained 98% monocytes at separation by Percoll. In preliminary experiments using the blood of five healthy controls, no significant loss of monocytes (<1%) dur-
ing preparation of MNC by Lymphoprep density gradient separ-
ation was observed. We thus conclude that monocytes were
FIG. 1. Representative example of flow cytometry plots and histograms showing expression of CD3, CD19, CD14, HLA-ABC, HLA-DR, CD80, CD86, and CD40 by monocytes obtained from a healthy control prepared with Percoll density gradient separation (as described in Materials and Methods). High percentages of monocytes expressing and high levels of CD14 on monocyte cell surface as reflected by mean fluorescence intensity were observed. Contamination of B (CD19+) and T (CD3+) cells in monocyte preparations did not exceed 10%. In concert with the characteristics of professional antigen-presenting cells, monocytes expressed high percentages and high levels of HLA-ABC, HLA-DR, and the costimulatory molecule CD86 and moderate percentages and levels of the costimulatory molecules CD40 and CD80 on monocyte cell surface. PerCP, peridinin chlorophyll protein.
isolated successfully. Twenty milliliters of blood typically yielded $1.5 \times 10^6$ to $2 \times 10^6$ monocytes.

High levels of IL-6-, IL-10-, IL-12-, and TNF-α-secreting monocytes were found in blood from healthy subjects and are augmented by in vitro stimulation with LPS. Monocytes are important secretors of IL-6, TNF-α, IL-10, and IL-12 among MNC (19, 21). Therefore, IL-6-, TNF-α-, IL-10-, and IL-12-secreting cells should be mainly confined to the low-density fraction, representing monocytes, as obtained by Percoll separation. To test this hypothesis, we performed ELISPOT assays on MNC, cells of the high-density fraction, and cells from the low-density fraction, prepared from the same blood samples. In parallel, the proportions of monocytes and B and T cells among MNC, high- and low-density fractions of the same samples were also analyzed. Higher numbers of cells secreting IL-6, IL-10, IL-12, and TNF-α were observed in the low-density fraction, representing monocytes, compared to MNC of the same sample ($P < 0.01$, $P < 0.01$, $P < 0.05$, and $P < 0.01$, respectively). Higher numbers of IL-6-, IL-10-, IL-12-, and TNF-α-secreting cells in the monocyte fraction compared to the high-density fraction, representing mainly B and T cells, were also observed ($P < 0.001$, $P < 0.01$, $P < 0.05$, and $P < 0.01$, respectively). In parallel, we observed high proportions of monocytes, which were confined to the low-density fraction (Fig. 2). We therefore conclude that monocytes, as obtained by Percoll separation, are confined to the low-density fraction and contain high numbers of cells secreting IL-6, IL-10, IL-12, and TNF-α.

LPS is the principal component of the outer membrane of gram-negative bacteria. Human monocytes are exquisitely sensitive to LPS and respond by expressing many inflammatory cytokines. To evaluate the in vitro effects of LPS on numbers of cytokine-secreting monocytes, monocytes prepared from 25 healthy subjects were used. We observed high levels of IL-6-, IL-10-, TNF-α ($P < 0.0001$ for all comparisons)- and IL-12 ($P < 0.001$)-secreting monocytes upon LPS stimulation compared to monocytes without such stimulation. We thus conclude that monocytes prepared by Percoll density gradient are capable of responding efficiently to exogenous stimuli, e.g., LPS (Fig. 3).

**Evaluation of optimal incubation time for monocyte ELISPOT assays.** Background staining of individual wells and levels of the cytokines produced by the individual cells are highly dependent on interactions between the individual cells, incubation period, etc. Changes in assay conditions such as incu-
FIG. 3. Numbers of IL-6 (A)-, TNF-α (B)-, IL-10 (C)-, and IL-12 (D)-secreting monocytes from 25 healthy subjects after culture in the absence (0) and in the presence of LPS. Arrows refer to median values.
bation period, therefore, are likely to influence the assay results and ease of discriminating spot from background staining considerably. Optimal incubation of ELISPOT plates, therefore, should result in high numbers of clearly defined dark spots against a light background. To determine the optimal incubation time, monocytes were incubated for 24, 48, and 72 h. Higher numbers of IL-6-, TNF-α-, IL-10-, and IL-12-secreting monocytes were observed after 24 h of incubation compared to 72 h of incubation (\(P < 0.05\), \(P < 0.001\), and not significant, respectively). Higher numbers of TNF-α (\(P < 0.05\)) and IL-10 (\(P < 0.05\)) were also found after 24 h compared to those after 48 h of incubation (Table 1). Spots were also easy to count after 24 h of incubation when size and clarity of spots were taken into consideration. In addition, background staining was minimal after 24 h of incubation (Fig. 4). Therefore, a 24-h incubation period was chosen for all cytokines under study for further experiments. In preliminary tests, incubation with secondary antibody either for 2 h at room temperature or overnight at 4°C resulted in similar numbers of monocytes secreting IL-6, IL-10, IL-12, and TNF-α (data not shown). We chose incubation of secondary antibody overnight at 4°C for further studies.

**Evaluation of optimal cell numbers for monocyte ELISPOT assays.** To detect cells actively secreting cytokines by ELISPOT assays, cells should be plated in a single cell layer. In addition, the sensitivity of the assay is critically dependent on numbers of cells employed per individual cell and frequency of cytokine-secreting cells within the sample. Thus, for each cytokine optimal cell numbers, i.e., the balance between application of a single layer of cells versus high numbers of spots, should be determined. To determine cell concentrations yielding optimal numbers and quality of spots, different numbers of monocytes were incubated per well. Based on previous kinetic experiments using MNC (19, 20), we chose the four concen-

### Table 1. Numbers of IL-6, TNF-α, IL-10 and IL-12 secreting cells per 10^5 monocytes from 10 healthy subjects

| Incubation period (h) | IL-6 Range | Mean (SD) | Median | TNF-α Range | Mean (SD) | Median | IL-10 Range | Mean (SD) | Median | IL-12 Range | Mean (SD) | Median |
|----------------------|------------|-----------|--------|-------------|-----------|--------|-------------|-----------|--------|-------------|-----------|--------|
| 24 (n=10)            | 4,550–54,400 | 13,343 (14,622) | 9,450   | 2,800–40,800 | 15,045 (11,155) | 13,750 | 5–530       | 182 (218) | 27     | 12–476      | 215 (171) | 199    |
| 48 (n=10)            | 2,875–27,200 | 9,527 (7,149) | 6,963   | 800–21,600  | 7,510 (6,215)   | 5,900  | 0–218       | 59 (82)   | 8      | 5–247       | 108 (75)  | 95     |
| 72 (n=10)            | 2,625–16,500 | 7,753 (5,290) | 5,288   | 450–13,900  | 4,885 (4,003)   | 4,275  | 0–15        | 7 (6)     | 8      | 3–160       | 72 (60)   | 70     |

*a* Monocytes were incubated for 24, 48, and 72 h and examined by ELISPOT assays. Incubation for 24 h yielded higher numbers and better quality of spots compared to incubation for 72 h for all cytokines under study. Higher numbers of TNF-α- and IL-10-secreting cells were observed after 24 h compared to 48 h of incubation. Since the incubation time of 24 h was found to yield high numbers of spots for the cytokines under study, and spots were easy to identify, the 24-h incubation was selected in all present and future experiments. \(P\) values for comparison of IL-6 values at 24 and 48 h, at 24 and 72 h, and at 48 and 72 h were not significant, \(<0.05\), and not significant, respectively. \(P\) values for comparison of TNF-α values were \(<0.05\), \(<0.001\), and not significant, respectively. \(P\) values for comparison of IL-10 values were \(<0.05\), \(<0.001\), and not significant, respectively. \(P\) values for comparison of IL-12 values were not significant, \(<0.01\), and not significant, respectively.

**FIG. 4.** Phenotypes of monocytes (Giemsas staining, \(\times 400\)) as prepared by Percoll density. (A) Monocytes are seen as large cells with large indented nuclei. Also shown are representative examples of spots representing IL-6 (B)-, TNF-α (C)-, IL-10 (D)-, and IL-12 (E)-secreting monocytes. Spots each representing a cell secreting cytokine appeared as clear dark-stained well-circumscribed areas. Stained artifacts were easily distinguished from the spots of secreted cytokine due to the size, stainability, and density of contaminated objects.
considered the application of 50,000 monocytes/200 μl/well for IL-6 and TNF-α. For IL-10, we chose the concentrations 50,000, 20,000, 10,000, and 5,000 monocytes/200 μl/well, and for IL-12, we chose 100,000, 80,000, 60,000, and 40,000 monocytes/200 μl/well. No statistical differences in numbers of spots per 10^5 monocytes were observed for TNF-α or for IL-6 different numbers of monocytes were applied. Spot size and spot quality though were considered optimal for 2,000 monocytes/200 μl/well for IL-10 and 1,000 monocytes/200 μl/well for TNF-α (Fig. 4 and 5). Numbers of IL-10-secreting cells per 10^5 monocytes were higher when 50,000 monocytes/200 μl/well were applied compared to 10,000 (P < 0.01) or 5,000 (P < 0.01) monocytes/well. Since numbers of IL-10-secreting monocytes were highest at the maximum concentration initially chosen, additional tests were performed. Eight healthy controls were studied, and cells were adjusted to 100,000 and 50,000 monocytes/200 μl/well. No statistical differences were observed in numbers of spots per 10^5 monocytes between cell concentrations of 100,000 and 50,000 monocytes/200 μl/well. We considered the application of 50,000 monocytes/200 μl/well for further studies. Also the numbers of IL-12-secreting cells per 10^5 monocytes were higher when 100,000 monocytes/200 μl/well were applied compared to 60,000 (P < 0.05) or 40,000 (P < 0.001) cells/200 μl/well. Since numbers of IL-12-secreting monocytes were highest at the maximum concentration chosen, monocytes prepared from eight healthy controls were studied. Upon adjusting cells to 200,000 and 100,000 cells per well, no differences in numbers of spots per 10^5 monocytes of IL-12-secreting monocytes were observed between these cell concentrations. We decided to select a concentration of 100,000 monocytes/200 μl/well to be used for further ELISPOT studies (Fig. 5).

**Interassay variability for monocyte ELISPOT assays.** To test interassay variability of monocyte ELISPOT assays, monocytes of five healthy subjects were prepared and applied to ELISPOT assays at two different occasions. Numbers of monocytes secreting IL-6 or IL-12 varied less than 10%, and numbers of monocytes secreting TNF-α or IL-10 varied less than 15% between the two samplings (Fig. 6).

**Detection of monocytes producing and secreting cytokine by ELISPOT assays and ELISA.** To compare different techniques for cytokine detection, monocytes from 18 healthy donors were prepared and analyzed for cytokine production by ELISPOT assays and ELISA in parallel. Using ELISPOT assays, cytokine-secreting monocytes could be detected in each specimen. High numbers of TNF-α- and IL-6-secreting cells were observed in healthy donors; IL-12- and IL-10-secreting monocytes were detected at a much lower frequency. Among healthy subjects, 5% ± 1% of the monocytes secreted IL-6, 7% ± 3% secreted TNF-α, 0.2% ± 0.1% secreted IL-10, and 0.1% ± 0.1% secreted IL-12 (Table 2). Variation between duplicates was <10% in ELISPOT assays. Secreted cytokines could also be detected by ELISA in all samples. Variation between duplicates for ELISA was 17% for TNF-α, 28% for IL-6, and 17% for IL-10; this variation between duplicates was much higher (P < 0.05, P < 0.001, and P < 0.0001, respectively) compared to ELISPOT assays. Variation between duplicates was similar for levels of IL-12 (10%) as measured by ELISA compared to numbers of IL-12-secreting monocytes by ELISPOT. Correlation between numbers of cytokine-secreting cells as measured by ELISPOT and levels of secreted cytokines by ELISA were observed for monocytes secreting TNF-α (r = 0.7; P < 0.01), IL-6 (r = 0.6; P < 0.01), and IL-12 (r = 0.5; P < 0.05) (Table 2).

**DISCUSSION**

The detection of cytokines is hampered by their biological properties, e.g., local secretion, rapid uptake and utilization, and short half-life (13). These properties have led to the de-
Development of numerous tools to detect transcribed, translated, or secreted cytokines. The usefulness of methods detecting either transcribed or translated proteins is limited since cytokines carry out their functions mainly after secretion of the protein. Therefore, detection of cytokines before their secretion might not be a reliable reflection of the released effector cytokine. Alternatively, secreted cytokines may be measured in body fluids using ELISA. The presence of cytokine binding proteins in serum might reduce the cytokine levels in biological fluids, making results obtained by ELISA less representative.

To date, the biological relevance of determining absolute quantities of cytokines has not been addressed. Cytokines may exert their influence with extremely variable levels of biologically active protein. Assessment of cytokine-secreting cells by ELISPOT circumvents most of these problems (8) and offers the advantage of close protein-capture antibody proximity and strong electrostatic interactions that circumvent paracrine and autocrine cytokine uptake of the cytokine (24). Thus, ELISPOT assay allows the detection of well-defined spots that clearly represent numbers of cytokine-secreting cells present in the circulation. In addition, high specificity combined with high sensitivity makes ELISPOT assays attractive tools in the evaluation of cytokines. A drawback of ELISPOT assays is the difficulty of establishing the assays for new cytokines due to scarce availability of suitable antibody pairs.

In the present study, ELISPOT assays were adapted to de-

![Graphs showing numbers of IL-6, TNF-α, IL-10, and IL-12 secreting cells per 10^5 monocytes from five healthy subjects (HC) sampled on two occasions. The variation in numbers of IL-6- and IL-12-secreting monocytes was less than 10% and less than 15% for IL-10 and TNF-α. Black bars represent first sampling.]

**TABLE 2.** Comparison of different methods of detecting spontaneous cytokine production by blood monocytes

| Method          | Production of indicated cytokine |
|-----------------|---------------------------------|
|                 | TNF-α   | IL-6    | IL-12   | IL-10   |
|                 | Range   | Mean (SD) | Median | Range   | Mean (SD) | Median | Range   | Mean (SD) | Median |
| ELISPOT (spots/100,000 monocytes) | 1,400–13,200 | 7,168 (3,203) | 6,800 | 2,800–7,950 | 5,039 (1,376) | 5,125 | 24–300 | 90 (86) | 61 |
| ELISA (ng/ml)   | 14,340–34,170 | 25,835 (5,954) | 27,755 | 861–18,610 | 8,317 (4,922) | 8,010 | 8,572 (5,030) | 6,640 |

a Blood monocytes from 18 healthy controls were prepared and analyzed for cytokine production by ELISPOT and ELISA in parallel. Correlations were observed between numbers of cells secreting (ELISPOT) and levels of secreted (ELISA) TNF-α, IL-6, and IL-12. For comparison of ELISPOT and ELISA results, r and P, respectively, were as follows: for TNF-α, 0.7 and <0.01; for IL-6, 0.6 and <0.01; for IL-12, 0.5 and <0.05; for IL-10, not significant.
tect and enumerate monocytes secreting selected cytokines. To obtain high yields of monocytes with high purity, we applied a modified two-step density gradient method (26). Many other methods of separating monocytes either lack the ability to yield sufficient numbers of cells of high purity or require expensive equipment and extensive separation time. Alternatively, pure populations of CD14+ monocytes are obtained by different sorting strategies. However, since CD14+ monocytes represent up to 25% of the monocytes in healthy donors, the CD14+ population might not represent the properties of the whole monocyte population. Density gradient separation of monocytes as used in the present study was relatively quick (less than 4 h) and yielded monocytes with a purity of >90%. Most of the monocytes were recovered by Percoll separation, leading us to conclude that no major selection of a subpopulation of monocytes occurred during separation. Yields obtained by this method were typically 1.5 × 10^6 to 2.0 × 10^6 monocytes per 20 ml of blood.

We adapted ELISPOT assays to detect monocytes secreting IL-6, TNF-α, IL-10, and IL-12. These cytokines are able to shift the immune response either towards a Th1 type via IL-12, thereby facilitating the production of cytokines such as TNF-α and IL-6, or to promote the production of Th2-related cytokines such as IL-4 by IL-10 (15). A tight regulation of these four cytokines, therefore, keeps the balance and decides whether Th1 or Th2 will predominate in immune reactions (5, 9, 22, 23). LPS is the principal component of the outer membrane of gram-negative bacteria. Humans have evolved to detect low levels of LPS to combat infections. Monocytes orchestrate the innate immune response to LPS by expressing a variety of inflammatory cytokines that include TNF-α (12). In the present study, we evaluated the in vitro stimulatory capacity of LPS on numbers of IL-6-, IL-10-, IL-12-, and TNF-α-secreting monocytes. We observed augmented numbers of monocytes secreting all of the cytokines under study upon LPS stimulation. The present findings thus may make ELISPOT assays a useful tool in studying changes in levels of cytokine secretion in monocytes secreting IL-6 under physiological conditions in BALB/c mice. J. Immunol. 159:2375–2378.

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