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Determination of cytokine co-expression in individual splenic CD4$^+$ and CD8$^+$ T cells from influenza virus-immune mice

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SUMMARY

We have studied the patterns of interleukin-2 (IL-2), IL-4 and interferon-γ (IFN-γ) co-expression displayed by individual splenic CD4$^+$ and CD8$^+$ T cells in response to influenza virus immunization. Unseparated spleen cells obtained from mice intraperitoneally (i.p.) injected with A/PR8 (H1N1) influenza virus (PR8) were cultured for 24 hr in the presence of ultraviolet-inactivated PR8. As controls, cultures of both naive spleen cells stimulated with PR8 or of immune cells lacking the inactivated virus were used. The frequencies of CD4$^+$ and CD8$^+$ T cells expressing IL-2, IL-4 and IFN-γ were determined by three-colour flow cytometric analysis of fixed and saponin-permeabilized cells fluorescent-stained for either CD4 or CD8 surface molecules and for one of the following combinations of two intracellular cytokines: IL-2/IL-4, IL-2/IFN-γ and IL-4/IFN-γ. The results showed that immunization with influenza virus induces in both CD4$^+$ and CD8$^+$ T cells a heterogeneity of cytokine response patterns that do not follow the type 1/type 2 polarized response model, but with substantial differences between the two populations. In fact, the analysis of the phenotypes of virus-immune CD8$^+$ T cells revealed similar significant proportions of cells either expressing any one of the three cytokines or co-expressing combinations of them (i.e. IL-4/IL-2, IL-4/IFN-γ and IL-2/IFN-γ), whereas immune CD4$^+$ T cells were seen to express almost exclusively a single cytokine per cell. The observed patterns of cytokine production suggest that influenza virus immunization induces the expression of a type 0 cytokine pattern at both population and single cell levels in CD8$^+$ T cells and exclusively at the population level in CD4$^+$ T cells.

INTRODUCTION

Exposure to various infectious agents can stimulate different cytokine production patterns in both CD4$^+$ and CD8$^+$ T cells (reviewed in refs. 1, 2). Originally, two functional subsets of CD4$^+$ T cells were described, based on the cytokines produced by these cells.[3] T helper 1 (Th1) cells, characterized by the secretion of interleukin-2 (IL-2), interferon-γ (IFN-γ) and lymphotoxins, induce cellular immunity against intracellular bacteria and parasites; while Th2 cells, characterized by the production of IL-4, IL-5, IL-10 and IL-13, promote a humoral response against extracellular pathogens.[2–4] A third population, Th0, of CD4$^+$ T cells which are able to produce both Th1 and Th2 cytokines was also recognized and probably represents the majority of circulating CD4$^+$ T cells.[3] Later, it was shown that CD8$^+$ T cells can also secrete combinations of cytokines analogous, but not identical, to Th1 or Th2 CD4$^+$ T cells during various infections both in humans and in mice.[5–7] These two types of cytokine production were termed type 1 and type 2, respectively. Further studies, however, indicated that many immune responses involve the production by T cells of both types of cytokines and are not dominated by any one pattern.[8] In agreement with the above results we have recently reported[9] that intraperitoneal (i.p.) immunization of mice with influenza virus did not induce type 1/type 2 polarized T-cell cytokine production. We assayed, by flow cytometric analysis of intracellular cytokines and surface antigen expression, the distribution of IL-2-, IL-4-, or IFN-γ-producing CD4$^+$ and CD8$^+$ T cells in *ex vivo* influenza virus-immune spleen cells restimulated *in vitro* with the same virus. We found the presence in both T-cell populations of significant percentages of cells capable of producing one of the three cytokines. This study, however, provided information on the cytokine-response patterns of immune T cells exclusively at the population level, since the methodology used enabled the determination of only one intracellular cytokine per cell at a time.

A further important step to understand the complexity of

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Abbreviations: IFN, interferon; IL, interleukin; PR8, A/PR8 (H1N1) influenza virus; Th, T helper.

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the T-cell cytokine response is the analysis of the combinations of the cytokine production at the single-cell level. Studies using various experimental models, showed different cytokine profiles in both individual $CD4^+$ and $CD8^+$ T cells. Experiments performed, for example, on clones of $CD4^+$ and $CD8^+$ T cells derived from a mixed lymphocyte reaction (MLR)[10] or on $CD4^+$ T cells primed *in vivo* with keyhole limpet haemocyanin (KLH),[11] showed that type 1 and type 2 cytokines can be co-expressed in the individual T cells and suggested the possibility that any one cytokine is produced according to a continuous, rather than a discrete, pattern.[8] In contrast, other research[12] indicated that in *in vitro* bacterial antigen-stimulated $CD4^+$ T cells the secretions of IFN-γ and IL-4 were mutually exclusive: but in some cells IL-10 was co-expressed with either IL-4 or IFN-γ. These and other results[2] suggest the existence of patterns of single-cell cytokine expression other than those of type 1/type 2 and indicate that different activation stimuli can induce specific expression patterns.

In the present study we have determined, by simultaneous flow cytometric analysis of the expression of a surface marker and two intracellular cytokines per cell, the pattern of IL-2, IL-4 and IFN-γ co-expression in individual splenic $CD4^+$ and $CD8^+$ T cells from influenza virus-immune mice. We found that influenza virus immunization induced the differentiation of subsets of both $CD4^+$ and $CD8^+$ T cells, each expressing exclusively IL-2, IL-4, or IFN-γ, and of a subset (or subsets) of $CD8^+$ T cells co-expressing different cytokines. No dominant type 1 or type 2 response was observed in the two T-cell subsets both at population and single-cell levels.

MATERIALS AND METHODS

Animals

Male BALB/c mice (5–7 weeks) purchased from Charles River Italia (Italy) and attested to be negative for antibodies to murine hepatitis virus, Sendai virus and mycoplasma by enzyme-linked immunosorbent assay (ELISA), were used.

Virus and immunization

A/PR8 (H1N1) influenza virus (PR8) was grown in the allantoic cavities of 10-day-old embryonated chicken eggs. Ultraviolet (UV)-inactivated PR8 was prepared by exposing virus to UV light (40 W, 254 nm, 8 cm distance) for 5 min. Mice to be immunized were i.p. injected with 500 haemagglutinating units (HAU)/animal of PR8.

Cell cultures

Spleens were removed 5 days after immunization. Splenocytes were plated into 24-well plates (Becton Dickinson, Lincoln Park, NJ) at 1×10^7/ml (2 ml/well) in culture medium [RPMI-1640 plus 10% fetal calf serum (FCS), 1% L-glutamine and 1% penicillin–streptomycin]. Cells were cultured alone or with 250 HAU/ml of UV-inactivated PR8 for 24 hr at 37° in humidified 5% CO_2 in air.

Three-colour flow cytometry for intracellular cytokines

The frequency of cytokine-producing $CD4^+$ and $CD8^+$ T cells was determined by the following method:[9,12] cultured cells were fixed in ice-cold phosphate-buffered saline (PBS) containing 4% paraformaldehyde for 20 min. After two washes in PBS the cells were resuspended in PBS containing 0·5% bovine serum albumin (BSA) and 0·01% NaN_3 and were aliquoted for staining at 1×10^6 cells. Then cy-chrome-conjugated rat anti-mouse CD4 monoclonal antibody (mAb; clone RM4-5) or rat anti-mouse CD8 mAb (clone 53-6.7) were added and the cells were incubated for 30 min at 4°. Cells were washed twice and resuspended in PBS containing 0·5% BSA, 0·01% NaN_3 and 0·3% saponin (Sigma, St. Louis, MO) for staining with rat anti-mouse cytokine mAb (0·25–1 mg mAb/10^6 cells) for 30 min at 4°. The following mAbs, all purchased from PharMingen (San Diego, CA), were used: fluorescein isothiocyanate (FITC) anti-IL-2 (clone JES6-5H4), phycoerythrin (PE) anti-IL-4 (clone 11B11), PE anti-IFN-γ (clone XGM1.2) and FITC anti-IFN-γ (clone XGM1.2). As negative controls, aliquots of cell suspensions were incubated with an irrelevant isotype-matched mAb conjugated to the same fluorochrome as the sample. Finally, after the incubation at 4° for 30 min with the above mAb, the cells were washed in PBS/BSA/NaN_3/saponin and resuspended in PBS/BSA/NaN_3 without saponin for cytofluorimetric analysis. A FACScan flow cytometer (Becton Dickinson, Mountain View, CA) equipped with a 15 mW argon ion laser and filter setting for FITC (530 nm), PE (585 nm) and cy-chrome, emitting in the deep red (>650 nm) was used. Ten thousand cells were computed in list mode and analysed using the FACScan Research Software (Becton Dickinson). Signals from light scatter channels and fluorescence-detecting photomultipliers (PMT) were collected in linear and logarithmic mode, respectively. The FL1, FL2 and FL3 channels were set up and compensated on a tight lymphocyte gate using tubes with a single-labelled strongly positive antibody. Dead cells were excluded by forward and side scatter gating. List mode files were then analysed for cytokine production by first defining an analysis gate on anti-CD4- or anti-CD8-positive T cells. Statistical markers were set using the irrelevant isotype-matched controls as reference. Typically, 1% positive cells were allowed beyond the statistical markers in these controls. Previous results[9,12] showed that no significant cytokine immunofluorescence is present in fixed but not saponin-permeabilized cells.

Quantification of cytokines in culture supernatants

Concentrations of IL-2, IL-4 and IFN-γ in the culture supernatants were measured by two-side sandwich ELISA, according to the method previously described,[13] using commercial reagents under conditions recommended by the manufacturer (PharMingen, San Diego, CA).

Statistical analysis

Statistical significance was determined using Student's *t*-test.

RESULTS

Expression of intracellular cytokines by individual $CD4^+$ T cells

Spleen cells obtained from mice i.p. injected with PR8 were cultured for 24 hr in the presence of UV-inactivated PR8. As control, cultures of naive spleen cells stimulated with PR8 and of immune cells lacking the inactivated virus were established. The frequencies of $CD4^+$ T cells expressing IL-2, IL-4 and IFN-γ were determined analysing, by three-colour flow cytometric procedure, fixed and saponin-permeabilized cells fluorescent stained for the CD4 surface molecule and for one of the following combinations of two intracytoplasmic cytokines:

IL-2/IL-4, IL-2/IFN-γ and IL-4/IFN-γ. Despite the lack of an amplification step, such as the presence of brefeldin A or monensin during *in vitro* stimulation, by using appropriate isotype controls we were able to detect in PR8-stimulated samples significant percentages of cytokine-positive cells. The cut-off point between positively and negatively stained cells was set at 1% of isotype controls.

As can be seen in Fig. 1, restimulation *in vitro* with PR8 of 5-day-primed splenocytes induced a statistically significant increase versus unstimulated controls of the number of individual $CD4^+$ T cells singly expressing IL-2, IL-4, or IFN-γ. In contrast, the frequency of PR8-stimulated $CD4^+$ T cells co-expressing IL-4 and IL-2 (Fig. 1a), IL-4 and IFN-γ (Fig. 1b) or IL-2 and IFN-γ (Fig. 1c) was found to be very low and not statistically different from unstimulated cells. Also, no statistically significant differences were found between the frequencies of $CD4^+$ T cells expressing each of the three cytokines. Furthermore no significant differences were found in the number of cells expressing a single cytokine as seen by analyzing for different pairs of cytokines, namely IL-4/IL-2 (Fig. 1a), IL-4/IFN-γ (Fig. 1b) and IL-2/IFN-γ (Fig. 1c). Finally, no significant percentages of cytokine-producing cells were observed in cultures of PR8-stimulated naive spleen cells (data not shown).

Expression of intracellular cytokines by individual $CD8^+$ T cells

The frequencies of IL-2-, IL-4- and IFN-γ-positive $CD8^+$ T cells were determined by three-colour flow cytometric analysis following the same experimental procedure used for $CD4^+$ T cells. Figure 2 shows that, in contrast to $CD4^+$ T cells, PR8 restimulation induced a significant increase versus unstimulated controls of the percentages of $CD8^+$ T cells both positive exclusively for IL-2, IL-4, or IFN-γ and double positive for IL-4/IL-2 (Fig. 2a), IL-4/IFN-γ (Fig. 2b) and IL-2/IFN-γ (Fig. 2c), respectively. Although no statistically significant differences were found between the percentages of $CD8^+$ T cells expressing single cytokines, the data seem to indicate a predominant induction of IFN-γ-producing cells by influenza virus followed by those producing IL-4 and IL-2. No significant differences were found in the percentages of cells singly expressing only a given cytokine by analysing different pairs of cytokines, namely IL-4/IL-2 (Fig. 2a), IL-4/IFN-γ (Fig. 2b) and IL-2/IFN-γ (Fig. 2c). Data also excluded a tendency for a hierarchy of cytokine co-expression as the frequencies of $CD8^+$ T cells co-expressing the different pairs of cytokines were not statistically different. Finally, no significant percentage of cytokine-producing $CD8^+$ T cells was observed in both unstimulated and PR8-stimulated naive spleen cells (data not shown).

Cytokine assay of supernatants from spleen cell cultures

To ascertain whether the *in vivo* immunization/*in vitro* restimulation procedure was effective in stimulating cytokine production in all the animals, the level of the different cytokines was measured by ELISA in the supernatants of the same spleen cell samples that were tested for intracellular cytokines. The results (Fig. 3) showed that the restimulation with PR8 of 5-day primed splenocytes obtained from individual mice induced the release of significant amounts of IL-2, IL-4 and

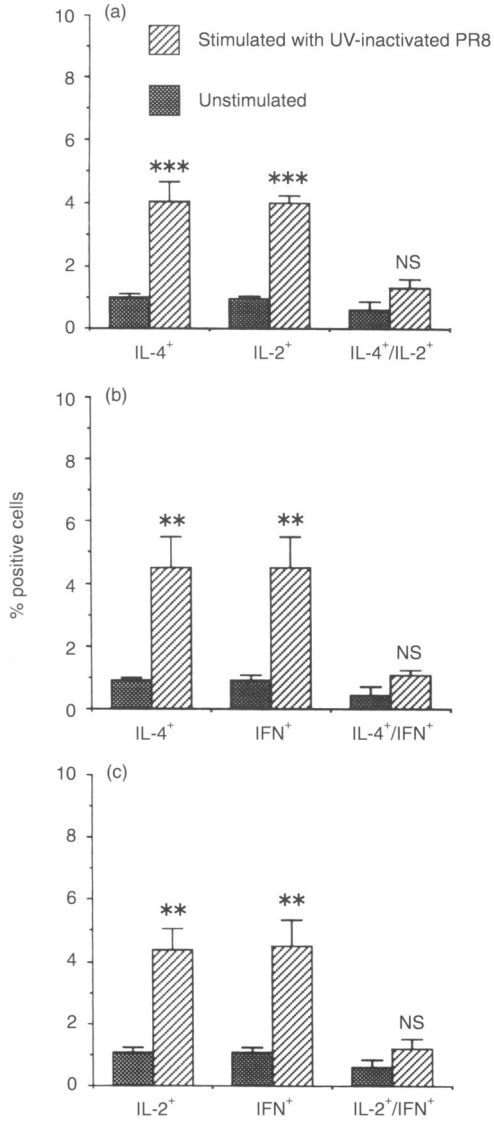

Figure 1. Frequency of $CD4^+$ T cells expressing only one of the cytokines IL-4, IL-2 and IFN-γ or co-expressing them, as determined by three-colour flow cytometry analysis. The percentage of cytokine-producing cells was evaluated on PR8-immunized animal-derived spleen cells both restimulated *in vitro* with UV-inactivated PR8 (hatched bars) or unstimulated (filled bars). All the samples were analysed for their correlate expression of (a) IL-4 versus IL-2, (b) IL-4 versus IFN-γ, (c) IL-2 versus IFN-γ. Samples were analysed by first gating on the CD4-positive population. The threshold for detection of positive cytokine-producing cells was set at 1% above the negative control consisting of cells treated with an isotype-specific antibody. Results are expressed as mean +SEM of at least six independent experiments. Statistical analysis (Student's *t*-test) was performed comparing PR8-stimulated cultures with unstimulated cultures. $^{**}P<0{\cdot}01$; $^{***}P<0{\cdot}001$; NS, not significant.

IFN-γ in all the animals tested. Concentrations of IL-2 ranged from 18·3 to 50·0 U/ml (mean ± SE 30·6 ± 2·54); IL-4 ranged from 6·4 to 29 ng/ml (15·0 ± 1·64) and IFN-γ from 14·9 to 47·0 pg/ml (30·0 ± 2·55). Very low levels of spontaneous release of these cytokines were detected in some of the unstimulated cultures from primed animals (Fig. 3) but not from unprimed animals (data not shown).

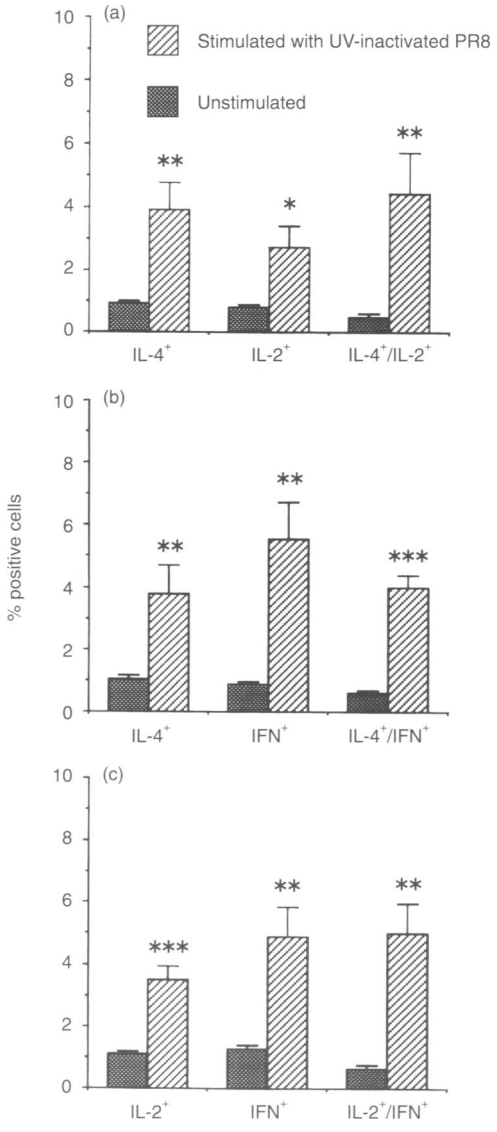

Figure 2. Frequency of $CD8^+$ T cells expressing only one of the cytokines IL-4, IL-2 and IFN-γ or co-expressing them, as determined by three-colour flow cytometry analysis. The percentage of cytokine-producing cells was evaluated on PR8-immunized animal-derived spleen cells both restimulated *in vitro* with UV-inactivated PR8 or unstimulated. All the samples were analysed for their correlate expression of (a) IL-4 versus IL-2, (b) IL-4 versus IFN-γ, and (c) IL-2 versus IFN-γ. Samples were analysed by first gating on the CD8-positive population. The threshold for detection of positive cytokine-producing cells was set at 1% above the negative control consisting of cells treated with an isotype-specific antibody. Results are expressed as mean +SEM of at least six independent experiments. Statistical analysis (Student's *t*-test) was performed comparing PR8-stimulated cultures with unstimulated cultures. $*P<0.05$; $**P<0.01$; $***P<0.001$.

DISCUSSION

The aim of this study was to define the cytokine phenotypes of individual spleen T cells obtained from influenza virus-immune mice and restimulated *in vitro* with the same virus. In a previous study[9] we demonstrated that, at population level, IL-2, IL-4 and IFN-γ were concurrently produced by both influenza virus-immune $CD4^+$ and $CD8^+$ T cells. Here we have determined, by simultaneous flow cytometric analysis of the expression of a surface marker and two intracellular cytokines, the IL-2, IL-4 and IFN-γ co-expression patterns of individual $CD4^+$ and $CD8^+$ T cells.

In accordance with previous results obtained in other experimental systems,[6,14] we found that primary immunization activated to produce cytokines only a limited, although significant, number of cells within both T-cell populations. The percentage of cytokine-positive cells (single- plus double-positive cells) was however, higher in $CD8^+$ T-cell than in $CD4^+$ T-cell subset (see Figs 1 and 2) thus suggesting a preferential activation of the $CD8^+$ T-cell population by influenza virus. Significant titres of all the three cytokines were detected in the supernatants of cultures of spleen cells obtained from all the immunized animals thus confirming the effectiveness of our immunization schedule. The cytokine co-expression patterns at the single cell level demonstrated that immunization with influenza virus induces an heterogeneity of cytokine response patterns, that do not follow the type 1/type 2 model, in both $CD4^+$ and $CD8^+$ T cells, but with substantial differences between the two populations. Immune $CD4^+$ T cells were seen to express almost exclusively a single cytokine per cell. In fact, we found significant percentages (versus naive $CD4^+$ T cells) of these cells expressing only IL-2 or IL-4 or IFN-γ and very low not significant percentages of cells co-expressing any combination of them. In contrast, the analysis of the phenotypes of virus-immune $CD8^+$ T cells revealed similar significant proportions of cells either expressing any one of the three cytokines or co-expressing combinations of them (i.e. IL-4/IL-2, IL-4/IFN-γ and IL-2/IFN-γ).

The marked tendency toward the production by individual immune $CD4^+$ T cells of a single cytokine per cell we observed in the present study is in agreement with previous studies[14] which reported that in primary response the co-expression by individual $CD4^+$ T cells of any pairs of cytokines represents a rare event. This suggested that primary antigenic stimulation generates a type 0 phenotype at population level rather than at single cell level.[14] Our results seem to confirm this hypothesis.

Interestingly, our data indicate that upon primary immunization with influenza virus, $CD8^+$ T cells can express, differently from $CD4^+$ T cells, a type 0 phenotype also at single cell level. In fact, the frequencies of $CD8^+$ T cells co-expressing IL-4/IL-2, IL-4/IFN-γ or IL-2/IFN-γ were found to be not statistically different, thus suggesting that the cells identified as double positive belong to a single subset of type 0 cells co-expressing all the three cytokines. It is important to note that we evaluated the cytokine profiles of both T-cell populations in spleen cells obtained from the same animal, in the same conditions of activation, at the same time after restimulation with influenza virus, in the presence of the same complexity of immunocompetent cells and at comparable assay sensitivity. Therefore, we can reasonably exclude that the observed differences in the cytokine co-expression pattern between individual immune $CD4^+$ and $CD8^+$ T cells could depend on differences in the experimental conditions used for induction and evaluation of cytokine production in the two T-cell subsets

Whether the different patterns of cytokine expression observed in *ex vivo* virus immune $CD4^+$ and $CD8^+$ T cells at 24 hr after *in vitro* restimulation reflect real differences *in vivo*

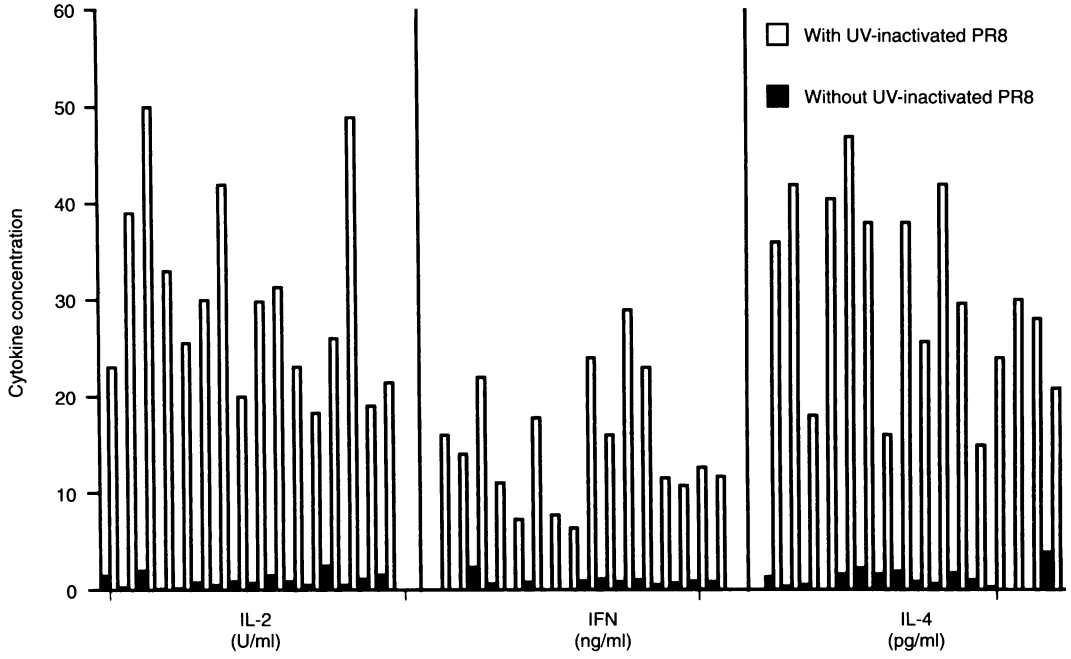

Figure 3. *In vitro* production of cytokines by influenza virus-primed spleen cells of individual mice. The levels of IL-2, IL-4 and IFN-γ were determined by ELISA in culture supernatants of PR8-immunized animal-derived spleen cells cultured for 24 hr in the presence or absence of UV-inactivated PR8. Each bar represents the results obtained with spleen cells from an individual mouse. A total of 16 mice were included in these experiments.

is not known. In fact, they may correspond either to distinct effector populations and different stages of development, or to transient responses induced by the particular stimulation conditions. However, the experimental conditions used in this study make it reasonable to assume the physiological relevance of these observations. In fact, we have used a naturally occurring, antigen-specific stimulus, rather than polyclonal stimuli like anti-CD3 or phytohaemmaglutinin, to obtain a 'physiological' production of cytokines and restimulation of spleen cells in bulk cultures to provide conditions for possible cross-regulatory activities by the different T-cell subsets through the cytokines produced by them. In addition, we obtained significant percentages of all the different $CD4^+$ and $CD8^+$ T-cell subsets by restimulating immune spleen cells exclusively with virus, that is without the use of further experimental interventions, like addition of cytokines to bulk cultures or derivation of clones in the presence of exogenously added cytokines, known to enhance differentiation toward particular types of cytokine-producing cells.[15–17]

The physiological significance of the differences in cytokine co-expression patterns showed upon influenza virus stimulation by $CD4^+$ and $CD8^+$ T cells awaits further analysis. However, an increasing amount of data suggest the existence of a link between the co-ordinate expression of certain cytokines and the effector function of T cells activated by different antigens.[16–20] Several previous studies clearly indicated that both individual $CD4^+$ and $CD8^+$ T cells can display heterogeneous cytokine profiles under different conditions of activation.[2,16] For example, short-term antigen-specific clones[10] and single $CD4^+$ T cells derived from KLH-immunized animals[11] showed a random co-expression of type 1 and type 2 cytokines, whereas bacterial antigen-stimulated $CD4^+$ T cells showed mutually exclusive intracellular synthesis of IFN-γ and IL-4.[12] Moreover, *in situ* hybridization studies of cytokine mRNA levels in cloned T-cell populations suggest that there exists heterogeneity of response also within clonal T-cell populations.[14,21] Similarly, earlier studies indicated that under different experimental conditions $CD8^+$ T cells can differentiate into polarized type 1 and type 2 subsets,[2,7,16,17] into subpopulations having polarized cytokine profiles distinct from type 1/type 2 patterns[22] or even into cells virtually secreting every combination of cytokines.[9] At the moment, however, very little information on the biological functions of the $CD4^+$ and $CD8^+$ T-cell subsets identified by the different cytokine profiles is available.

In conclusion, the data presented here show, in accordance with previous results in other systems of T-cell activation, that primary stimulation with influenza virus induces the activation of a small but significant number of both $CD4^+$ and $CD8^+$ T cells with heterogeneous cytokine profiles that do not follow a type 1 or type 2 pattern. They additionally suggest that influenza virus immunization induces the expression of a type 0 cytokine pattern, at both population and single cell level in $CD8^+$ T cells and exclusively at population level in $CD4^+$ T cells. Thus IL-2, IL-4 and IFN-γ can be secreted by both subsets, whereas there are substantial differences between $CD4^+$ and $CD8^+$ T cells in the cytokine secretion pattern of individual cells.

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