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IL-6 Increases Primed Cell Expansion and Survival

Irina Rochman,* William E. Paul,[†] and S. Z. Ben-Sasson^{1*†}

Cytochrome *c*-specific CD4 T cells from transgenic donors transferred to syngeneic B10.A mice expand more vigorously upon immunization if exogenous IL-6 is provided during the initial phase of immunization. The resultant increase in the frequency and number of Ag-specific cells is observed in the blood, lymph nodes, spleen, liver, and lung and persists for at least 3 mo. Treatment of immunized recipients with anti-IL-6 or use of IL-6 knockout recipients reduced the frequency of Ag-specific CD4 T cells during a comparable period, indicating that IL-6 is physiologically involved in the expansion of memory and/or effector cells and thus in the persistence of memory. IL-6 did not alter the duration of Ag-presenting activity. Both CFSE dilution studies and labeling with BrdU indicated that IL-6 does not effect proliferative rates of responding CD4 T cells. By contrast, annexin V staining was diminished in responding cells from the IL-6-treated animals, particularly among those cells that had undergone five or more divisions. These results indicate that IL-6 reduces the level of apoptosis among Ag-stimulated cells; thus, it plays a central role in determining numbers of memory and/or effector CD4 T cells in response to immunization over extended periods. *The Journal of Immunology*, 2005, 174: 4761–4767.

The conditions under which naive T cells encounter Ag profoundly affect the outcome of priming. Considerable attention has been focused on the nature and state of maturation of the dendritic cells (DC)² that present Ag to naive T cells. The degree of maturity of these cells, as reflected in their array of cell surface molecules and secreted cytokines and chemokines, can determine whether the stimulated T cells undergo a response leading to apoptosis or anergy or alternatively develop into effector and/or memory T cells (1–3). The pattern of cytokines produced by the DC, most notably whether IL-12 is made, plays an important role in the polarization of the responding T cells (4).

Other cytokines have been reported to control the survival and proliferation of T cells. IL-7 and IL-15 are important regulators of the expansion of CD8 cells (5–8), and IL-7 has been implicated in homeostasis of CD4 cells (9, 10) and in generation of CD4 memory cells (11, 12). The presence of IL-4 at the time of priming has been reported to strikingly enhance the numbers of CD8 memory T cells long after the priming event (13).

We have previously shown that the presence or absence of specific cytokines at the time of in vitro priming can profoundly affect the overall expansion of the responding T cell population, largely by regulating the survival of those cells. The cytokines that were most effective in leading to a robust expansion of responding naive T cells in vitro were IL-6 and IL-4, with IL-12 being somewhat less active. IL-2, IL-7, and IL-15 were not effective (14).

IL-6 is a pleotropic cytokine that regulates many aspects of immune responses, hemopoiesis, acute phase responses, and inflammation (15). It is critical for the development of lupus in NZB ×

NZW F₁ mice (16). Pristane-treated IL-6-deficient mice express lower levels of IgG anti-DNA Abs and milder renal disease than wild-type mice (17). IL-6-deficient mice have a deficit in the induction of experimental autoimmune encephalomyelitis (EAE) (18, 19), in the development of collagen-induced arthritis (20, 21), experimental autoimmune myasthenia gravis (22), and autoimmune myocarditis (23). Treatment with anti-IL-6 Abs blocks induction of inflammatory bowel disease in mice (24, 25). Indeed, IL-6 exerts antiapoptotic activity on a wide variety of cells (26–32); in particular, in vitro apoptosis of both naive (33) and recently activated T cells is inhibited by IL-6 (14, 34–38). Here we explore the relative importance of IL-6 present at the time of priming on in vivo responses of a defined population of naive CD4 T cells encountering their cognate Ag. We show that in the presence of added IL-6 during a 7-day period of priming, there is a greater degree of expansion of cells initially encountering their cognate Ag and that the numbers of the resultant primed cells are substantially enhanced for periods as long as 90 days. Furthermore, the absence of IL-6, achieved either by using IL-6 knockout mice or by treatment with anti-IL-6 at the time of priming, strikingly reduces the Ag-driven expansion of CD4 T cells and the resultant number of long term memory and/or effector T cells. Thus, IL-6 plays an important physiological role in the regulation of induction of Ag specific responses and in controlling the numbers of Ag-specific cells that result from the priming.

Materials and Methods

Mice

Female 5C.C7/RAG-2^{-/-} B10.A mice (line 94), 5C.C7/RAG-2^{-/-}/CD45.1 B10.A mice (line 205), B10.A CD3 ϵ ^{-/-} mice, B10.A CD3 ϵ ^{-/-} IL-6^{-/-} mice, and syngeneic female B10.A mice were obtained from Taconic Farms. The mice were maintained in our animal facilities under pathogen-free conditions and were used at 6–12 wk of age.

Cytokines and Abs

Mouse IL-6 was purchased from PeproTech. Neutralizing anti-IL-6 (MP5-20F3) Abs, fluorochrome-conjugated anti-V α 11 (RR8-1), anti-V β 3 (KJ25), anti-CD4 (L3T4), anti-CD43 (S7), anti-CD44 (IM7), anti-CD45.1 (A20), anti-CD62L (MEL-14), anti-IL-2 (JES6-5H4), anti-IL-4 (11B11), and anti-IFN- γ (XMG1.2) Abs and streptavidin were purchased from BD Pharmingen. Fluorochrome-conjugated annexin V and BrdU flow kits were purchased from BD Pharmingen.

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² Abbreviations used in this paper: DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis.

In vivo priming of adoptively transferred cells

Cells ($1.5\text{--}2 \times 10^7$) from lymph nodes and spleens of line 94 mice were injected i.p. into normal syngeneic B10.A mice (3–5 mice for each experimental group). Miniosmotic pumps (Durect) containing 1 mg of pigeon cytochrome *c* (Sigma-Aldrich) with or without IL-6 (100 μg) in HBSS were implanted in recipient B10.A mice 7–10 days later. The miniosmotic pumps were removed 7 days after implantation. In some of the experiments, the line 94 cells were injected i.p. 24 h after pump implantation.

Cell division analysis

Lymph node cells from line 205 mice were washed twice in PBS and resuspended in PBS at a concentration of 2×10^7 cells/ml. An equal volume of freshly diluted 2.5 μM CFSE (Molecular Probes) in PBS was added for 8 min at room temperature; staining was stopped by the addition of an equal volume of FCS. Cells were immediately washed three times in RPMI 1640 containing 10% FCS and injected i.p. into syngeneic B10.A mice. After 7 days, the recipient mice were implanted with miniosmotic pumps containing cytochrome *c* or cytochrome *c* plus IL-6. Three days later, lymph nodes and spleens were removed, stained with APC-anti-CD4 and PE-anti-CD45.1. The percent of cells in various CFSE peaks among $\text{CD4}^+\text{CD45.1}^+$ cells was determined by analysis with a FACSCalibur and CellQuest software (BD Biosciences). From the distribution of the proportion of cells in each CFSE peak, the expected cell yield if there had been no cell death was calculated according to the formula:

$$n = n_0 \sum_{i=0}^k \frac{f_i}{2^i} \quad (1)$$

in which n = expected cell yield (assuming no death); n_0 = initial cell number; f_i = fraction of the cells at a given i cycle; and k = highest cell cycle.

Cell cycle analysis in vitro

Lymph node cells from unprimed line 94 mice or from mice that had been immunized with cytochrome *c* in the presence of IL-6 2 mo earlier were labeled with CFSE and incubated in 24-well plates (5×10^5 cells/well) with APC (2.5×10^5 T-depleted spleen cells) from normal B10.A mice and 0.1 μM cytochrome *c* peptide (88–104). The cells were harvested after 104 h in culture and stained with PE anti-V β 3 and APC anti-CD4 Abs. The percentage of cells in various CFSE peaks among the $\text{V}\beta 3^+\text{CD4}^+$ cells was determined by FACSCalibur analysis with CellQuest software (Becton Dickinson).

Monitoring the donor transgenic CD4 cells in the recipient organs

At specified times, mice were bled, and the percentage of $\text{V}\alpha 11/\text{V}\beta 3$ cells among the CD4^+ cells in the blood was determined by staining with FITC-conjugated anti-V $\alpha 11$, PE-conjugated anti-V $\beta 3$, and APC-conjugated anti-CD4 Abs in the presence of anti-Fc γ R/III Abs (2.4G2) for 30 min at 4°C followed by flow cytometry analysis with a FACSCalibur and CellQuest software. The number of donor transgenic cells in the lymphoid organs of the recipient mice was determined by multiplying the viable CD4^+ cell number in the lymph nodes and in spleen by the percentage of CD4^+ cells that are $\text{V}\alpha 11/\text{V}\beta 3$ or $\text{CD45.1}/\text{CFSE}$ positive.

Annexin V staining

Externalization of phosphatidylserine was detected by FITC-conjugated annexin V (BD Pharmingen). Briefly, cells were labeled with PE-conjugated anti-V $\alpha 11$ and biotin-conjugated anti-V $\beta 3$ followed by APC-avidin, as previously described. The stained cells were washed with annexin V binding buffer (BD Pharmingen) and incubated with FITC-annexin V at room temperature in 100 μl of annexin V binding buffer. After 15 min, 400 μl of annexin V binding buffer were added, and the cells were analyzed immediately by FACSCalibur and CellQuest software.

BrdU labeling

At various times after immunization, mice were injected i.p. with 1 mg of BrdU (Sigma-Aldrich) and then twice more at 2-h intervals. The mice were killed 2 h after the third BrdU injection, and the lymphoid organs were harvested. Isolated cells were stained with PE-conjugated anti-V $\alpha 11$ and biotin-conjugated anti-V $\beta 3$ followed by APC-avidin, as previously described. After staining for surface markers, the cells were fixed and stained with FITC-anti-BrdU (BD Pharmingen) according to the manufacturer's instructions. The stained cells were analyzed by FACSCalibur and CellQuest software.

Determination of cytokine-producing potential

Splenocytes were taken from B10.A mice that had previously received line 94 cells and had been immunized with cytochrome *c* or cytochrome *c* plus IL-6 3 mo earlier. Control mice received line 94 cells only. After the RBC were lysed with ammonium chloride potassium buffer, the cells were incubated with 1 μM cytochrome *c* peptide 88–104 in culture medium containing IL-2 (10 U/ml) and neutralizing anti-IFN- γ , anti-IL-4, and anti-IL-12 Abs (10 $\mu\text{g}/\text{ml}$ each). At the indicated times, the cells were transferred to wells coated with anti-CD3/anti-CD28 Abs for 6 h. Monensin (2 μM) was added to the wells for the last 2 h. The cells were removed from the wells by gentle pipeting, fixed for 10 min at room temperature with 4% paraformaldehyde, and permeabilized with 0.1% saponin in PBS containing 0.1% BSA. The permeabilized cells were stained with PE anti-V $\alpha 11$, FITC anti-V $\beta 3$, and either APC anti-IL-2, APC anti-IL-4 or APC anti-IFN- γ Abs in the presence of anti-Fc γ R/III Abs. The percentage of cytokine producing cells among the $\text{V}\alpha 11/\text{V}\beta 3$ population was determined by FACSCalibur analysis with CellQuest software.

Results

IL-6 enhances the frequency of in vivo-primed CD4 T cells

Line 94 cells were transferred to B10.A mice. Two weeks later, miniosmotic pumps containing cytochrome *c* were implanted. In some cases, IL-6 (100 μg) was included in the pump. The pumps were removed 7 days after implantation. The frequency of $\text{V}\alpha 11/\text{V}\beta 3$ cells among CD4^+ T cells in the blood of the recipients was measured from day –2 (with regard to pump implantation) to day +60. As shown in Fig. 1A, the frequency of transferred cells rose rapidly, peaked on days 11–12, and then gradually declined. Mice that had received IL-6 showed frequencies of TCR-transgenic cells that were more than twice those in mice that received only Ag over the course of the experiment. Indeed, from day 34 onward, the IL-6 group had frequencies of TCR transgenic cells 3- to 4-fold that of the Ag-only group.

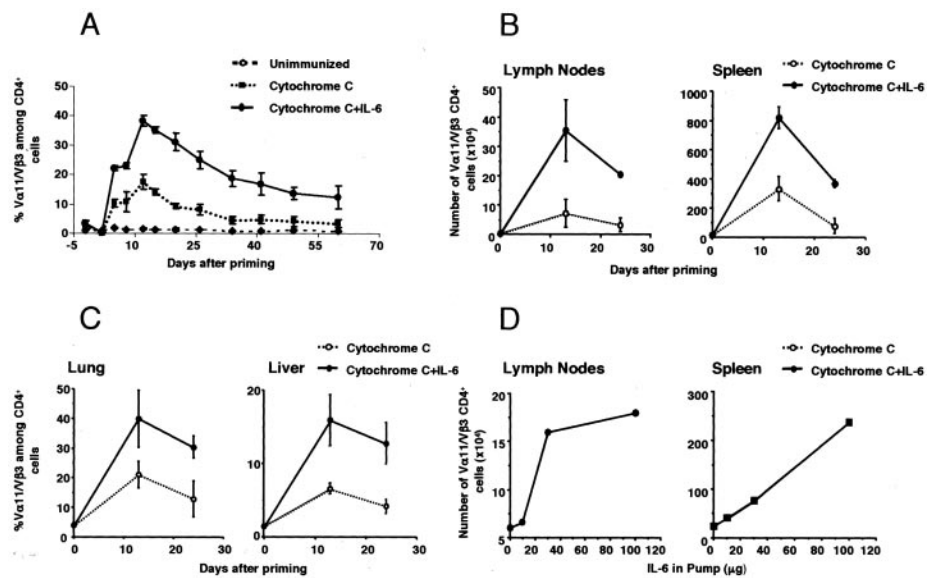
A comparable degree of enhancement was observed in lymphoid tissue (spleen and lymph nodes; Fig. 1B) and in parenchymal organs (lung and liver) (Fig. 1C). When measured in absolute numbers of cells, there were 6 times more TCR-transgenic cells in the IL-6 group at the peak of the response in the lymph node and 3 times more in the spleen. The differences in numbers of cells was the same or greater at day 72 (Fig. 1D); the effect of IL-6 was dose dependent; 10 $\mu\text{g}/\text{pump}$ was much less effective than 100 μg .

Cells in primed mice have properties of memory cells

The transferred $\text{V}\alpha 11/\text{V}\beta 3$ cells that had been stimulated with cytochrome *c* with or without added IL-6 were similar in their expression of high levels of CD44 and low levels of CD62L (Fig. 2A). In addition, their expression of CD43, which has been associated with memory cells (39), was also similar. As another test of their memory status, we examined the Ag sensitivity of the primed cells. More than 53% of the cells from the primed donors underwent six or more divisions in response to a 0.1 μM concentration of the CC peptide. A population of naive cells tested in parallel replicated more slowly; 26% went through six or more divisions and 10% failed to divide whereas virtually all of the cells from the primed donors had divided two times or more (Fig. 2B).

Three months after priming, the capacity of the cells to produce IL-2, IL-4, and IFN- γ was assessed, both acutely and after restimulation under conditions that prevent either Th1 or Th2 differentiation (i.e., in the presence of anti-IL-4, anti-IL-12, and anti-IFN- γ). Very little cytokine was produced within 6 h of stimulation with immobilized anti-CD3 and anti-CD28 (3–7% of the cells produced IL-2, and 0–2% of the cells secreted IFN- γ ; (data not shown). However, after restimulation of spleen cells with cytochrome *c* peptide, the transgenic cells from both the IL-6 group and the non-IL-6 group showed striking IL-4-producing capacity (Fig. 2C). At 92 h, 20–25% of the cells produced IL-4. By

FIGURE 1. IL-6 enhances the frequency of in vivo-primed CD4 T cells. Naive line 94 cells were transferred into B10.A mice. Two weeks (A) or 1 day (B–D) later, the mice were immunized by implantation of miniosmotic pumps containing cytochrome *c* (1 mg) with or without rIL-6 (100 μg). At the indicated times after priming, cells from the lymphoid organs (B and D) and from the lung and liver (C) were stained for CD4, Vα11, and Vβ3 as described in *Materials and Methods*. The number of CD4⁺Vα11⁺Vβ3⁺ T cells in peripheral lymph nodes and spleen was determined as described in *Materials and Methods* (B and D). D, Numbers of CD4⁺Vα11⁺Vβ3⁺ cells in lymph nodes and spleens of mice 72 days after priming with cytochrome *c* (1 mg) and various concentrations of rIL-6 in the pumps.



contrast, among TCR-transgenic cells that had been transferred to B10.A mice that were not immunized, fewer than 5% produced IL-4 at 92 h. The IL-2-producing capacity of the three groups of cells was relatively similar. Few cells produced IFN-γ; no differences were observed between the IL-6 and non-IL-6 groups or indeed between the nonprimed and primed groups.

Endogenous IL-6 contributes to primed cell numbers

To test whether endogenous IL-6 exerted an effect on the expansion and the maintenance of primed cell numbers, mice that had received

cytochrome *c*-containing miniosmotic pumps and into which line 94 cells had been transferred received anti-IL-6 on the day of pump implantation and 4 days later. Those animals that received anti-IL-6 had 3- to 6-fold fewer transgenic cells in lymph nodes and spleen on day 52 (Fig. 3A). We also transferred line 94 cells into in IL-6/CD3ε knockout mice and their IL-6-sufficient CD3ε knockout littermates. Miniosmotic pumps containing cytochrome *c* were implanted on the same day. The IL-6 knockout animals had 2- to 3.5-fold fewer line 94 cells on day 82 than did the littermates (Fig. 3B). Thus, IL-6 plays an important role in determining the expansion/survival of primed cells.

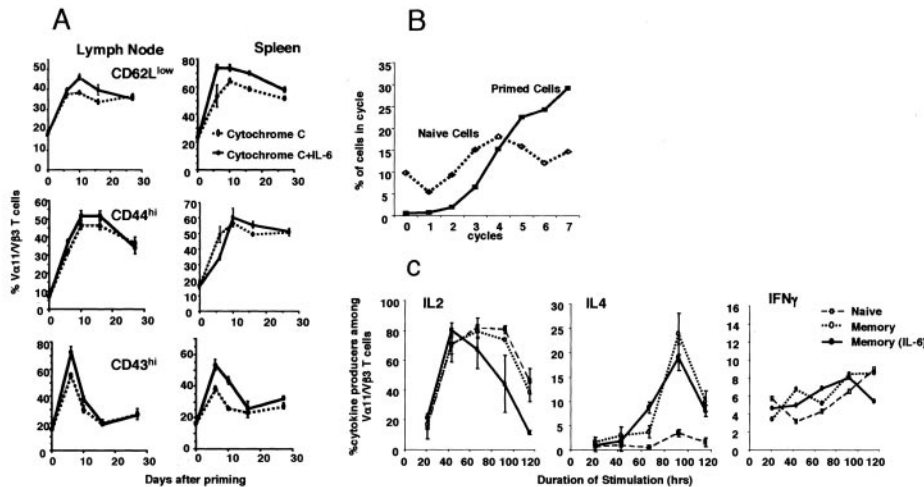


FIGURE 2. Transferred CD4 lymphocytes acquire properties of memory and/or effector cells. A, Miniosmotic pumps containing cytochrome *c* (1 mg) with or without rIL-6 (100 μg) were implanted into normal B10.A mice. One day after pump implantation, the mice were adoptively transferred with naive line 94 cells. At the indicated time points, cells from the lymph nodes and spleen of B10.A mice were collected and stained for Vα11/Vβ3 in combination with anti-CD43, anti-CD44 or anti-CD62L. B, Lymph node cells were obtained from normal line 94 mice or from line 94 mice that were primed for 2 mo earlier with cytochrome *c* (1 mg) and rIL-6 (100 μg). Cells were labeled with CFSE. The CFSE-labeled cells were cultured in 24-well plates with APC and 0.1 μM cytochrome *c* peptide. At 104 h of culture, the cells were harvested and stained with anti-Vβ3 and anti-CD4 Abs. The percentage of cells in various CFSE peaks among the Vβ3⁺CD4⁺ cells was determined by analysis with a FACSCalibur and CellQuest software (BD Biosciences). C, Splenocytes were obtained 88 days postimmunization from B10.A mice that had received line 94 cells and had been primed with cytochrome *c* with or without rIL-6. Naive line 94 T cells were obtained from the spleens of B10.A mice that had been adoptively transferred with line 94 cells 2 wk after transfer without subsequent immunization. The cells were activated in vitro with cytochrome *c* peptide and analyzed for the production of IL-2, IL-4, and IFN-γ by intracellular staining as described in *Materials and Methods*.

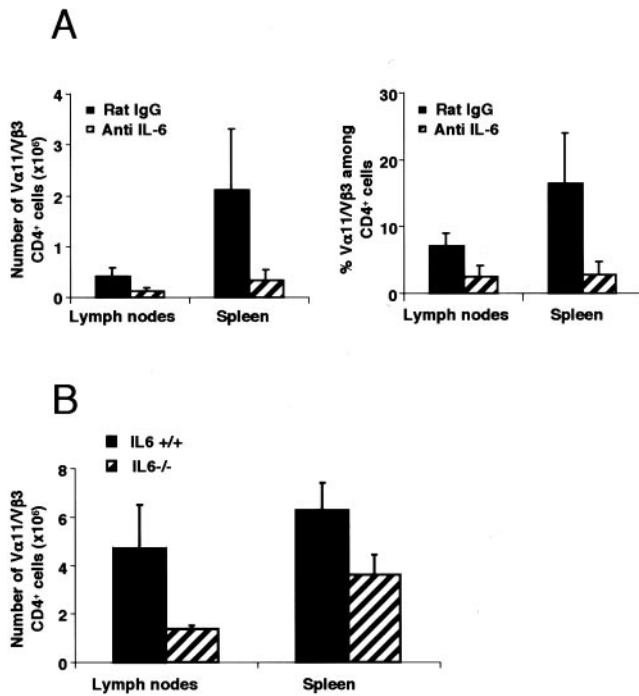


FIGURE 3. Endogenous IL-6 increases the yield of long-lived memory cells. *A*, B10.A recipients of line 94 cells were immunized by implantation of miniosmotic pumps containing 1 mg cytochrome *c*. On the same day and 4 days later, the mice were injected (i.v.) with either 100 μ g of neutralizing rat anti-IL-6 or rat IgG. On day 52, the number of CD4⁺V α 11⁺V β 3⁺ cells in the lymph nodes and spleens was determined. *B*, IL-6^{-/-} mice (B10.A/Ai-[KO]CD3- ϵ [KO]IL-6) and IL-6^{+/+} control mice (B10.A/SgSnAi-[KO]CD3- ϵ) were recipients of line 94 cells. They were immunized by implantation of miniosmotic pumps containing 1 mg of cytochrome *c*. On day 82, the number of CD4⁺V α 11⁺V β 3⁺ cells in the lymph nodes and spleens was determined.

IL-6 does not prolong the period of effective Ag presentation

To exclude the possibility that IL-6 might mediate its function by prolonging the period that APCs could continue to stimulate responses of TCR-transgenic cells, we implanted miniosmotic pumps containing cytochrome *c* with or without IL-6 and delayed the transfer of line 94 cells for 8, 11, or 14 days, respectively. In these experiments, the transferred cells were labeled with CFSE. The pattern of proliferation in the recipient mice that were immunized in the presence or absence of IL-6 was the same on each day, implying that IL-6 does not prolong the period of effective Ag presentation (Fig. 4).

IL-6 does not have a major effect on TCR-stimulated proliferation

CFSE-labeled line 94 cells were transferred into B10.A mice bearing miniosmotic pumps containing cytochrome *c* with or without IL-6. Three days later, the lymph nodes and spleen of the mice that received IL-6 had 2- to 3-fold more TCR-transgenic cells than did the control mice (Fig. 5A). However, when the pattern of proliferation was examined by flow cytometric analysis of CFSE dilution, there was a very modest difference that could have accounted for increases in cell yield of 1.1- and 1.3-fold, in the lymph node and spleen, respectively (Fig. 5B). Similarly, when the fraction of cells in S phase was measured by injection of BrdU 6 h before sacrifice, no differences were observed on days 4, 8, and 18 after pump implantation (Fig. 5C). Thus, the greater expansion of the transgenic cells in response to Ag in the presence of IL-6 cannot be accounted for an increase in proliferative rate.

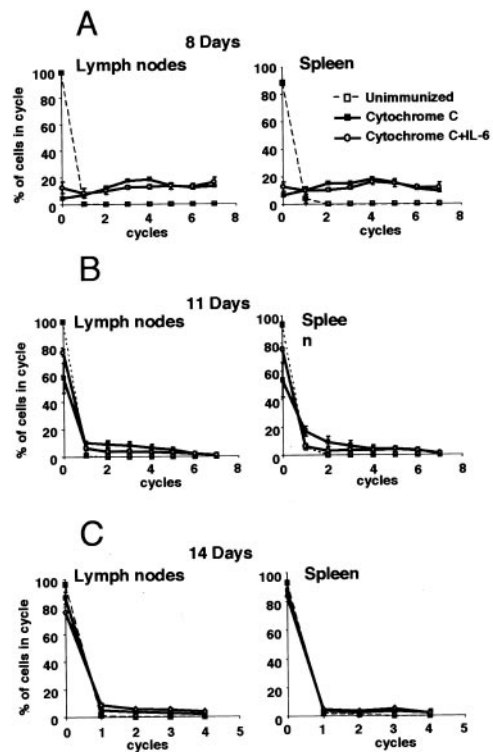


FIGURE 4. IL-6 does not prolong the period of Ag presentation. Miniosmotic pumps containing cytochrome *c* (1 mg) with or without rIL6 (100 μ g) were implanted into normal B10.A mice. On the 8th (*A*), 11th (*B*), or 14th day (*C*) after pump implantation, line 94 cells labeled with CFSE were injected i.p. into these mice. Unimmunized B10.A recipients served as a control. Four (*A*), six (*B*), or nine days (*C*) later, the replication of the CFSE-labeled CD4⁺V β 3⁺ T cells in lymph nodes and spleens was evaluated by FACS of CFSE dilution.

IL-6 diminishes apoptosis among Ag-stimulated transgenic cells

On days 4, 9, 16, and 26 after pump implantation, freshly isolated spleen cells were stained with annexin V. There was a substantial increase in the frequency of stained TCR-transgenic cells among those harvested from mice that had not received IL-6 (Fig. 6A). The difference in apoptosis on day 3 after pump implantation was found principally in cells that had undergone five or more cell divisions (Fig. 6B), suggesting that IL-6 was particularly important in determining the survival of cells that had been strongly stimulated by Ag.

Discussion

The current data, demonstrating a striking effect of IL-6 in enhancing the in vivo expansion and survival of Ag-stimulated CD4 T cells, give insight into a mechanism through which cells of the innate immune system regulate adaptive responses. IL-6 is produced by various cell types including macrophages, DCs, and B cells (40–42). Bacterial (43, 44) and viral (45, 46) infection are major inducers of IL-6 production. In particular, stimulation of macrophages and DCs with LPS (47–49) or CpG-containing oligodeoxynucleotides (50, 51) stimulate IL-6 production. The capacity of microbial pathogens to induce the production of IL-6 emphasize how this cytokine could play an important role in the primary immune response.

In vitro, IL-6 allows naive CD4 T cells stimulated with immobilized anti-CD3 and anti-CD28 in the presence of IL-2 to continue to expand beyond day 5, a time when rigorously purified naive cells cultured without IL-6 begin to diminish strikingly in number

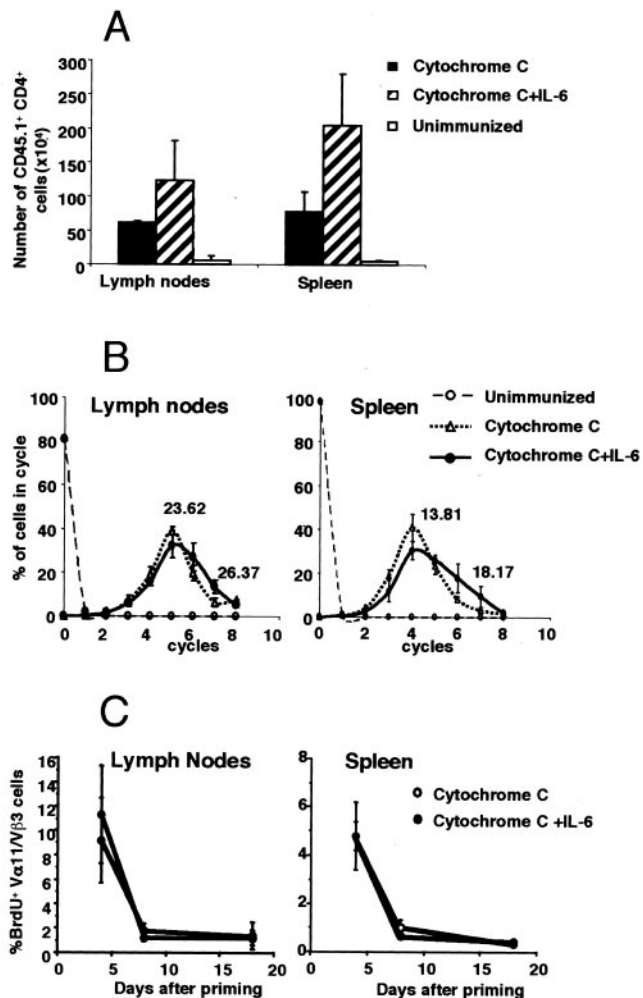


FIGURE 5. IL-6 does not have a major effect on the replication rate of the Ag-primed cells. CFSE-labeled line 205 cells were transferred into B10.A mice. Seven days later, miniosmotic pumps containing cytochrome *c* (1 mg) with or without rIL-6 (100 μ g) were implanted. Unimmunized B10.A mice that received naive line 94 cells served as a control. Three days after priming, the number of CD4⁺CD45.1⁺ T cells in lymph nodes and spleens was determined as specified in *Materials and Methods* (A). The CFSE-labeled CD45.1⁺CD4⁺ cells were analyzed for their rate of replication by CFSE dilution (B), and the expected relative cell yield of the cells indicated on the graph was calculated as described in *Materials and Methods*. Naive line 94 cells were transferred to normal B10.A mice 1 day after priming with miniosmotic pumps containing 1 mg of cytochrome *c* with or without rIL-6 (100 μ g). At the indicated times, mice were injected with 1 mg of BrdU three times at 2-h intervals and then sacrificed. Cells from lymph nodes and spleens were stained with anti-BrdU and the percent of BrdU⁺ cells among the V α 11V β 3 population presented in C was determined.

(14); similar results with anti-CD3 stimulation have also been reported (34–36). In our previous studies (14), the division rates of the stimulated cells, as judged by CFSE dilution, were not altered by IL-6, indicating that its major effect was to enhance survival. The effect of IL-6 was also tested in cells stimulated with their cognate Ag and different populations of APC. The effect of adding IL-6 compared with a control that received anti-IL-6 was also striking, implying that IL-6 could play a role in determining the overall expansion of naive T cells even in the presence of competent APC. Similarly, IL-6 has been reported to enhance in vitro survival of human CD4 cells that had been stimulated in vivo (37, 38), although the in vitro survival of murine CD4 T cells that were

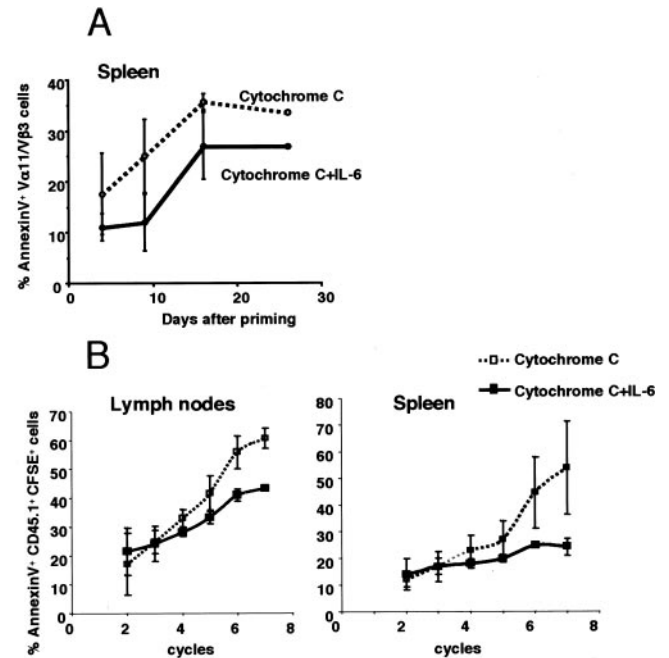


FIGURE 6. IL-6 diminishes the apoptosis of Ag-stimulated CD4 T cells. A, Miniosmotic pumps containing cytochrome *c* (1 mg) with or without rIL-6 (100 μ g) were implanted into normal B10.A mice. One day after immunization, line 94 cells were transferred into the mice. At the indicated time points, the percent of FITC-annexin V⁺ cells among the V α 11⁺V β 3⁺ cells from the spleens was determined by FACS. B, CFSE-labeled line 205 cells were transferred to B10.A mice 1 week before implantation of pumps containing cytochrome *c* (1 mg) with or without rIL-6 (100 μ g). Three days later, the percent of annexin V⁺ cells among the replicating CFSE-labeled CD45.1⁺ population in the lymph nodes and spleens was evaluated by staining with APC-annexin V.

activated in vivo by staphylococcal enterotoxin B was not enhanced by IL-6 in culture (33).

In apparent contradiction of the majority of these in vitro studies, Pape et al. (52) failed to observe an effect of exogenous IL-6 on a primary in vivo CD4 T cell response to OVA in a system in which TCR-transgenic T cells were transferred to congenic recipients. However, the total amount of IL-6 administered during these experiments was 1.5 μ g/mouse; we observed that >10 μ g of IL-6, provided continuously during a 7-day period, was needed to observe an effect on priming. Thus, we would argue that the discrepancy between the results of Pape et al. (52) and of those presented in this paper can be accounted for by the amounts of IL-6 available in the primed animal. More importantly, our results demonstrate that neutralizing IL-6 during the primary response by administering anti-IL-6 on days 0 and 4 substantially diminished the number of Ag-specific cells present on day 52, indicating that endogenous IL-6 present during the priming period played an important role in survival/expansion of cells. The physiological importance of IL-6 was further supported by the observation that priming TCR-transgenic transferred to IL-6^{-/-} mice was impaired.

The pathophysiological importance of IL-6 is emphasized by studies in IL-6 knockout mice. These mice have been reported to fail to control infection with vaccinia, *Listeria monocytogenes*, *Toxoplasma gondii*, and pathogenic *Escherichia coli* (53–56), and their T cell-dependent Ab response against vesicular stomatitis virus was impaired (53).

IL-6 knockout mice are resistant to several experimental autoimmune diseases, including the induction of EAE in response to

immunization with myelin oligodendrocyte glycoprotein (18, 19), collagen-induced arthritis (20, 21), autoimmune myasthenia gravis (22), and autoimmune myocarditis (23). Treatment of mice with anti-IL-6 Ab lessened the development of autoimmunity in lupus-prone NZB \times NZW F₁ mice (16) and reduced the severity of EAE (57). Anti-IL-6R Ab prevented induction of collagen-induced arthritis (58) and of colitis on transfer of CD45RB^{high} CD4 T cells (24). This treatment reversed established inflammatory bowel disease in IL-10 knockout mice and in trinitrobenzene sulfonic acid-induced colitis in normal mice (25). Anti-IL-6R Ab is in advanced clinical trials for the treatment of human rheumatoid arthritis (59), and a clinical trial is under way for treatment of SLE patients with anti-IL-6R Ab (URL- NLM identifier NCT00046774).

The mechanism through which the absence of IL-6 diminishes autoimmune disease has not been extensively investigated. There is evidence that blocking IL-6 in mice with inflammatory bowel disease caused apoptosis of lamina propria T cells (25, 60, 61). IL-6 knockout mice failed to develop MOG-specific effector T cells in the EAE model described above (62), and in the myocarditis model, CD4 T cells from the IL-6 knockout proliferated poorly in response to specific Ag (23).

Here we have shown that IL-6 has a striking effect on the numbers of Ag-specific CD4 T cells at the height of the response. This in keeping with unpublished observations we have made (S. Z. Ben-Sasson and W. E. Paul, unpublished observations) in IL-6-treated TCR-transgenic mice. These animals showed a striking induction of CD4 T cells on immunization with their cognate Ag, cytochrome *c*; ~70% of their CD4 T cells were capable of producing IL-4 on day 16 after immunization, compared with ~35% in mice immunized in the absence of added IL-6. Moreover, early exposure to IL-6 resulted in a long term (85-day) increase in the frequency of memory (CD44^{bright}CD62L^{low}) CD4 T cells from 30% in the control group to 70% in the IL-6-treated mice. Strikingly, this effect is seen long after the removal on day 7 of the IL-6-containing miniosmotic pump. These results are in keeping with those reported in this communication.

In vitro studies indicated that the IL-6 effect could not be accounted for by increase proliferative rates, implying that IL-6 rescued activated CD4 T cells from apoptosis. This effect of IL-6 in promoting survival is not unique to T cells. A variety of other cell types have been shown to survive better in the presence of IL-6, including plasma cells, liver cells, neurons, mast cells, endothelial cells, pancreatic β cells, and various tumor cells (26–32). Although the direct effect of IL-6 as a survival factor has been shown directly in many cell types including CD4 cells, we cannot exclude the possibility that some of its effects on the enhanced expansion of activated CD4 cells are mediated indirectly by restraining the inhibitory activity of the regulatory CD4⁺CD25⁺ cells in vivo as proposed by Pasare and Medzhitov (63).

Our in vivo analysis also strongly implicates cell survival, not increased proliferative rate, as the mechanism through which IL-6 enhances cell yield in immunized mice. CFSE dilution of the transferred cells reveals essentially no difference in division history of the responding cells. A 6-h pulse with BrdU at various times between day 2 and day 26 showed no difference in the rate of entry into S phase of the two groups. Because the absolute number of CD4 T cells strikingly increases in all organs tested, including liver and lungs, this can only be explained by a diminished death rate in the presence of excess IL-6 and an enhanced death rate when IL-6 is neutralized or absent. Indeed, the increased binding of annexin V, particularly among the fast replicating cells, strongly implies that IL-6 mediates its effect by protecting the T cells from apoptotic death.

These results imply that IL-6 may have potential value in enhancing the magnitude of specific immune responses. Whether one could foresee vaccine trials with IL-6 as a human adjuvant will very much depend on the toxicity profile of IL-6 and most particularly on the amount of the cytokine that may be required. However, in instances in which heat-killed vaccines are used, the relatively poor induction and short term of T cell responses might be substantially improved through the use of IL-6 at the time of priming. This would be most appropriate in instances of therapeutic vaccination or in those situations in which the infectious agent carries a very high risk, such as HIV. The anticipated introduction of anti-IL-6R as a treatment for rheumatoid arthritis re-emphasizes the importance of this agent. However, the relative importance of anti-IL-6 in blocking the inflammatory cascade, operating in a manner similar to that of anti-TNF- α , and its possible effect on memory T cells, must be investigated.

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Disclosures

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