T Cell Stimulation In Vivo by Lipopolysaccharide (LPS)

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Summary

Lipopolysaccharide (LPS) from gram-negative bacteria causes polyclonal activation of B cells and stimulation of macrophages and other APC. We show here that, under in vivo conditions, LPS also induces strong stimulation of T cells. As manifested by CD69 upregulation, LPS injection stimulates both CD4 and $CD8^+$ T cells, and, at high doses, stimulates naive ($CD44^{lo}$) cells as well as memory ($CD44^{hi}$) cells. However, in terms of cell division, the response of T cells after LPS injection is limited to the $CD44^{hi}$ subset of $CD8^+$ cells. In contrast with B cells, proliferative responses of $CD44^{hi}$ $CD8^+$ cells require only very low doses of LPS (10 ng). Based on studies with LPS-nonresponder and gene-knockout mice, LPS-induced proliferation of $CD44^{hi}$ $CD8^+$ cells appears to operate via an indirect pathway involving LPS stimulation of APC and release of type I (α, β) interferon (IFN-I). Similar selective stimulation of $CD44^{hi}$ $CD8^+$ cells occurs in viral infections and after injection of IFN-I, implying a common mechanism. Hence, intermittent exposure to pathogens (gram-negative bacteria and viruses) could contribute to the high background proliferation of memory–phenotype $CD8^+$ cells found in normal animals.

$CD4^+$ and $CD8^+$ T cells can be subdivided into naive and memory cells on the basis of various surface markers, especially CD44, CD45RB, and CD62L (L-selectin) (1). Thus, naive T cells express a $CD44^{lo}$ $CD45RB^{hi}$ $CD62L^{hi}$ phenotype, whereas most memory cells are $CD44^{hi}$ $CD45RB^{lo}$ $CD62L^{lo}$. T cells with a naive phenotype are resting cells and show a very slow turnover as measured by incorporation of the DNA precursor, bromodeoxyuridine (BrdU) (2, 3). By contrast, the majority of memory–phenotype cells have a rapid turnover.

T cells with a memory–phenotype are presumed to be the progeny of naive T cells responding to specific antigen. Why these cells show a high rate of turnover is unclear. The most obvious possibility is that memory–phenotype cells are engaged in chronic, low level proliferative responses to residual depots of specific antigen and/or to cross-reactive environmental antigens (4, 5). In addition to such TCR-mediated stimulation, memory–phenotype cells may also be subject to nonantigen–specific stimulation via cytokines. In support of this idea, infection of mice with viruses such as lymphocytic choriomeningitis virus (LCMV)[1] causes an intense T proliferative response, which appears to be predominantly nonantigen specific (6–8). This bystander response preferentially affects memory–phenotype ($CD44^{hi}$) $CD8^+$ cells and can be mimicked by injection of type I (α, β) interferon (IFN-I) and by Poly I:C, an inducer of IFN-I (9). These findings suggest that the constant proliferation of memory–phenotype T cells seen in normal animals may be mediated in part by IFN-I and other cytokines released during contact with various infectious agents. If so, any microorganism capable of stimulating IFN-I production in vivo would be expected to cause bystander stimulation of T cells.

To assess this possibility, we have examined the effects of injecting mice with LPS, a component of the cell wall of gram-negative bacteria (10). LPS (endotoxin) is well known for its capacity to cause polyclonal activation of B cells. However, in addition, LPS is strongly stimulatory for APC such as macrophages, and induces these cells to release various cytokines, e.g., IFN-I, TNF-α, and IL-12 (11–13). Hence, the production of these cytokines might be expected to stimulate T cells. In line with this prediction, evidence is presented that even small doses of LPS cause marked proliferation of $CD44^{hi}$ $CD8^+$ cells in vivo.

Materials and Methods

Mice and Treatments. C57BL/6J (B6) mice were purchased from either the rodent breeding colony at The Scripps Research Institute or The Jackson Laboratory (Bar Harbor, ME). C3H/HeOuJ, C3H/HeJ, B cell–deficient (μMT), and IFN-γ–deficient (IFN-$\gamma^{-/-}$) mice were purchased from The Jackson Laboratory. 129/SvEvTacfBR (129) mice were purchased from Taconic Farms (Germantown, NY). 129 background mice defective in IFN-α/β receptor function (IFN-$IR^{-/-}$) (14) were originally purchased from B&K Universal (North Humberside, UK) and were maintained and bred in the animal facility at The Scripps Research Institute. Where indicated, mice were injected intravenously with LPS (from *Escherichia coli* serotype 055:B5, prepared by trichloroacetic acid extraction) (Sigma Chemical Co., St. Louis, MO) or

[1]*Abbreviations used in this paper:* BrdU, bromodeoxyuridine; IFN-I, type I (α, β) interferon; LCMV, lymphocytic choriomeningitis virus.

Poly I:C (Sigma Chemical Co.) dissolved in 0.2 ml PBS. For bromodeoxyuridine (BrdU) treatment, mice were given sterile drinking water containing 0.8 mg/ml BrdU (Sigma Chemical Co.). BrdU-containing drinking water was changed daily.

Spleen Cell Transfer. Spleens from C3H/HeOuJ mice were disrupted using a tissue homogenizer and the resulting cell suspension was depleted of T cells by treatment with anti-CD8 and anti-CD4 mAbs plus complement (15). After antibody and complement treatment, cells were washed three times and resuspended in PBS. Cells were injected intravenously in a final volume of 0.5 ml (2.3×10^8 cells/recipient) into C3H/HeJ mice.

Flow Cytometry. mAbs used for cell surface staining were the following: anti-CD8–PE (GIBCO BRL, Gaithersburg, MD), anti-CD4–PE (Collaborative Biomedical Products, Bedford, MA), anti-Ly-6C–biotin (PharMingen, San Diego, CA), anti-B220–PE (PharMingen), anti-CD69–biotin (PharMingen), anti-CD44–FITC (PharMingen), and anti-CD44–biotin (IM7.8.1).

Biotinylated antibodies were detected with RED670–streptavidin (GIBCO BRL). Staining for BrdU with anti-BrdU–FITC (Becton Dickinson, Mountain View, CA) was done as described (3). Stained cells were analyzed on a FACScan® flow cytometer (Becton Dickinson).

Results

Effects of LPS on Normal Mice. To examine the effects of LPS on T cell turnover, normal adult C57BL/6 (B6) mice were injected intravenously with graded doses of LPS and immediately placed on BrdU water. After 3 d, suspensions of spleen and LN cells were stained for expression of cell surface markers, fixed, and then stained for BrdU incorporation (see Materials and Methods). The data shown below are representative of at least two separate experiments.

As expected, LPS injection caused significant proliferation of B cells, especially in spleen (Fig. 1). However, relative to the background proliferation in uninjected mice, BrdU incorporation by B cells was only seen after injection of relatively high doses of LPS (≥10 μg). For T cells, LPS injection had minimal effects on $CD4^+$ cells. Thus, even high doses of LPS had no effect on naive ($CD44^{lo}$) $CD4^+$ cells and caused only a slight increase in the high (30%) background rate of proliferation of memory–phenotype ($CD44^{hi}$) cells. Quite different results applied to $CD8^+$ cells. As for $CD4^+$ cells, LPS injection did not alter the slow turnover of $CD44^{lo}$ cells. However, even very low doses of LPS (10 ng) caused substantial proliferation of $CD44^{hi}$ $CD8^+$ cells, both in spleen and LN; with higher doses of LPS, BrdU labeling of $CD44^{hi}$ $CD8^+$ cells reached 70% (compared with 20% in uninjected mice). Significantly, LPS injection caused a considerable increase in Ly6C expression on $CD8^+$ cells. Upregulation of Ly6C expression on (total) $CD8^+$ cells is prominent in mice injected with Poly I:C and IFN-I and is reported to be controlled specifically by IFN-I (16). Hence, the upregulation of Ly6C on $CD8^+$ cells after LPS injection signified the production of IFN-I.

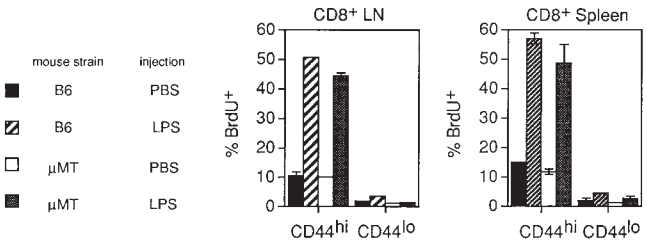

Figure 2. LPS induction of T cell proliferation in B cell–deficient mice. PBS or LPS (12.5 μg) was injected intravenously into B6 or μMT mice, and mice were given BrdU in their drinking water for 3 d. Percent BrdU labeling is shown for $CD8^+$ $CD44^{hi}$ and $CD8^+$ $CD44^{lo}$ LN (*left*) and spleen (*right*) cells. Data represent mean values from two mice (± SD).

Role of B Cells. To examine whether stimulation of $CD8^+$ T cells by LPS required B cells, we examined the effects of injecting LPS into B cell–deficient μMT mice. As shown in Fig. 2, BrdU labeling of $CD44^{hi}$ $CD8^+$ cells after LPS injection was almost as high in B6 μMT mice as in normal B6 mice. These data indicate that stimulation of $CD8^+$ cells by LPS does not require the presence of B cells.

T Cell Responses in LPS-nonresponder Mice. In the case of B cells, the C3H/HeJ strain of mice is largely refractory to stimulation by LPS (17). To test whether the LPS-nonresponder status of C3H/HeJ mice applies to T cells, we ex-

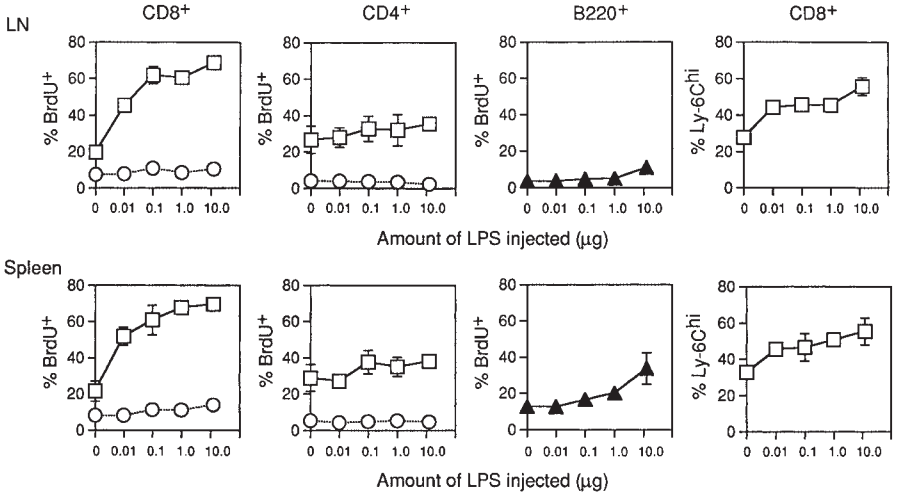

Figure 1. Effect of LPS injection on T and B cell proliferation in vivo. Graded doses of LPS were injected intravenously into B6 mice. Mice were immediately given BrdU in their drinking water, and LN and spleen cells were analyzed 3 d later. The three leftmost columns show percent BrdU labeling for $CD44^{hi}$ (□) and $CD44^{lo}$ (○) $CD8^+$ and $CD4^+$ cells and total $B220^+$ cells (▲), while the right hand panel shows the percentage of total $CD8^+$ cells expressing high levels of Ly-6C (Ly-$6C^{hi}$). The data represent mean values from two or three mice per point (± SD).

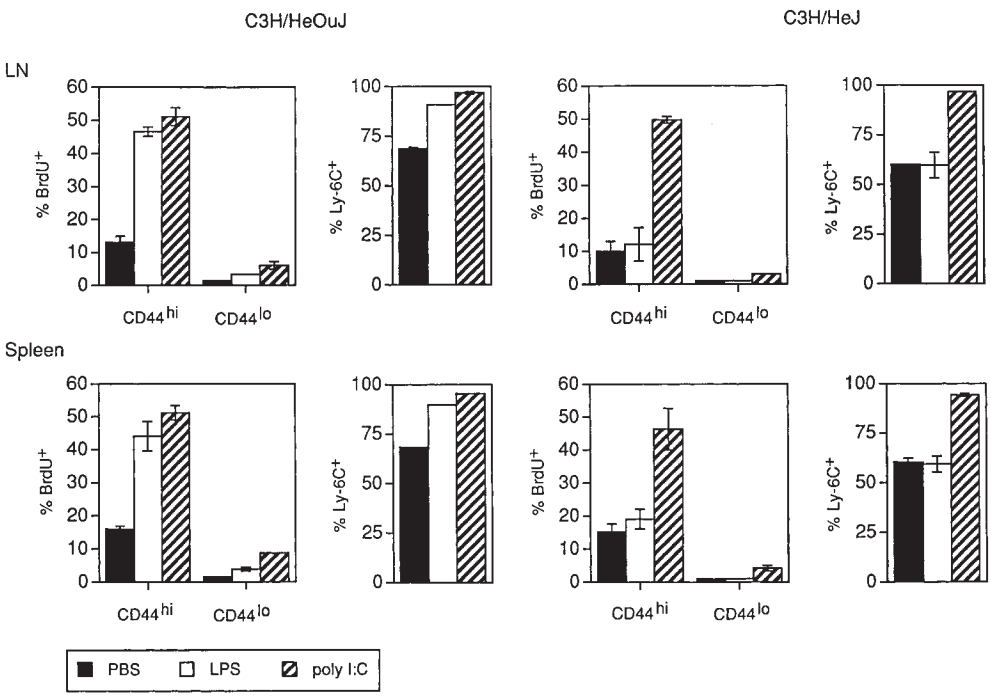

Figure 3. Failure of LPS to induce T cell proliferation in LPS-nonresponder mice. PBS (■), LPS (12.5 μg) (□), or Poly I:C (100 μg) (▨) was injected intravenously into C3H/HeOuJ (LPS-responder, *left*) or C3H/HeJ (LPS-nonresponder, *right*) mice. Mice were given BrdU in their drinking water for 3 d, then $CD8^+$ LN (*upper*) and spleen (*lower*) cells were assayed for BrdU labeling and Ly-6C expression. Data represent the mean values from two mice (± SD).

amined the effects of injecting LPS into C3H/HeJ versus LPS-responder C3H/HeOuJ mice; injection of Poly I:C was used as a control. As shown in Fig. 3, BrdU labeling of $CD44^{hi}$ $CD8^+$ cells after injection of a relatively high dose of LPS (12.5 μg) was prominent in C3H/HeOuJ mice but virtually undetectable in C3H/HeJ mice; similar findings applied to upregulation of Ly6C expression. By contrast, injection of Poly I:C caused BrdU labeling of $CD8^+$ cells and Ly6C upregulation in both strains of mice. These data indicate that the defective response of C3H/HeJ mice to LPS applies to T cells.

If T cell stimulation induced by LPS injection reflects cytokine production by APC rather than direct binding of LPS to T cells, reconstituting C3H/HeJ mice with normal LPS-responder APC would be expected to restore the response of T cells to LPS. To examine this question, C3H/HeJ mice were injected intravenously with large numbers of T-depleted spleen cells taken from normal C3H/HeOuJ mice ± LPS. As shown in Fig. 4, the presence of normal spleen APC at the time of LPS injection led to significant stimulation of the host C3H/HeJ $CD44^{hi}$ $CD8^+$ cells, especially in spleen. The failure of LPS-nonresponder T cells to proliferate after LPS injection could thus be restored by coinjecting normal APC.

Role of Receptors for IFN-I. The above findings suggest that stimulation of T cells after LPS injection requires a direct action of LPS on APC, presumably leading to the production of stimulatory cytokines. To examine whether IFN-I is required for LPS-induced T cell proliferation, we examined the effects of injecting LPS into mice lacking receptors for IFN-I (IFN-IR^- mice); mice lacking the gene for IFN-γ (IFN-γ^- mice) were used as a control. As shown in Fig. 5, injecting an intermediate dose of 1 μg of LPS caused prominent proliferation of $CD44^{hi}$ $CD8^+$ cells in IFN-γ^- hosts (Fig. 5 *B*) but virtually no proliferation in IFN-IR^- hosts (Fig. 5 *A*). However, a higher dose of 12.5 μg LPS did cause substantial proliferation of $CD8^+$ cells in IFN-IR^- mice, as well as in IFN-γ^- mice. These findings indicate that IFN-I production does play a crucial role in LPS stimulation of T cells, though only with a moderate dose of LPS. It should be noted that LPS-induced proliferation of B cells was not reduced in IFN-IR^- mice, implying that the response of B cells does not require IFN-I (data not shown).

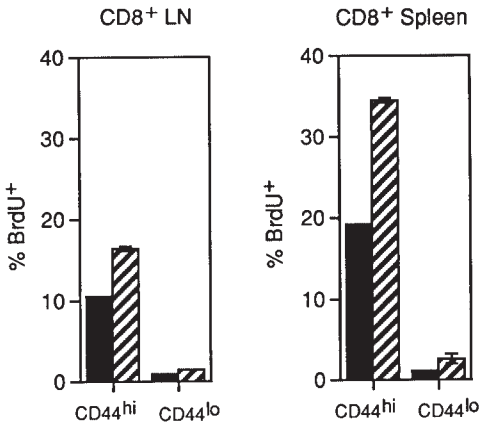

Figure 4. LPS induction of $CD8^+$ T cell proliferation in LPS-nonresponder mice supplemented with T-depleted spleen cells from LPS-responder mice. T-depleted C3H/HeOuJ spleen cells (2.3×10^8 cells/recipient) were injected intravenously into C3H/HeJ mice. 1 d later, the recipients were injected with PBS (■) or 12.5 μg LPS (▨) and then given BrdU water for 3 d. Data represent mean values from two mice (± SD).

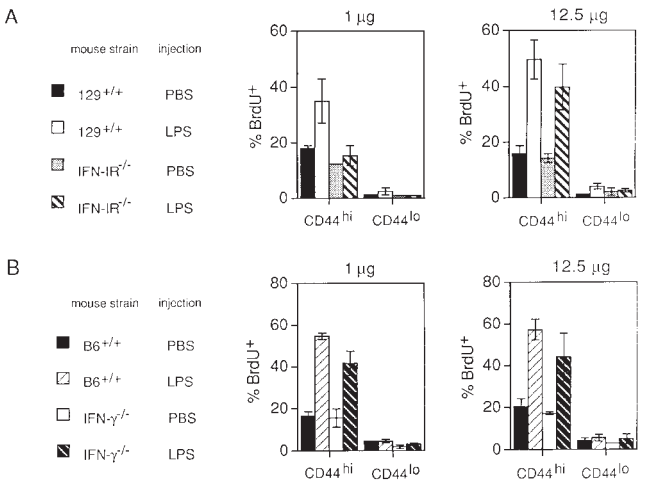

Figure 5. Role of IFN-I in LPS-induction of $CD8^+$ T cell proliferation. (*A*) Comparison of the T cell–proliferative response to LPS in control (129) mice versus mice deficient for the IFN-IR. (*B*) Comparison of the T cell proliferative response to LPS in control (B6) mice versus mice deficient for IFN-γ. 1 μg (*left*) or 12.5 μg (*right*) LPS was injected intravenously and mice were given BrdU water for 3 d. The results shown are for $CD8^+$ spleen cells. Data represent mean values for 2–4 mice per point (± SD).

Stimulation of Naive T Cells. In all of the above experiments, the capacity of LPS injection to stimulate T cells was restricted to the $CD44^{hi}$ subset of $CD8^+$ cells. However, this finding refers only to T cell proliferation. To examine other parameters of cell activation, T cells from LPS-injected mice were examined 1 d later for upregulation of CD69 expression, a highly sensitive indicator of T (and B) cell activation (18). The results shown in Fig. 6 *A* make three points. First, high doses of LPS caused upregulation of CD69 not only on $CD8^+$ cells and B cells (as expected from the BrdU labeling studies) but also on $CD4^+$ cells. Second, based on the results of injecting graded doses of LPS, the sensitivity of $CD8^+$ cells, B cells, and $CD4^+$ cells to LPS-induced CD69 upregulation seemed to be remarkably similar. Third, with high doses of LPS, CD69 upregulation applied to a high proportion (60–80%) of total $CD4^+$ and $CD8^+$ cells, suggesting that the LPS-stimulated cells included naive T cells. In support of this possibility, CD69 upregulation was clearly apparent on both $CD44^{lo}$ and $CD44^{hi}$ T cell subsets, both for $CD4^+$ cells and $CD8^+$ cells (Fig. 6 *B*). Although $CD44^{hi}$ cells were more sensitive to CD69 upregulation than $CD44^{lo}$ cells, injection of a high dose of LPS induced CD69 expression on >50% of $CD44^{lo}$ cells, both for $CD4^+$ and $CD8^+$ cells.

Discussion

Although LPS-induced proliferation of lymphoid cells is thought to be primarily restricted to B cells, there are a number of reports that LPS can stimulate T cells. Thus, it is well established that LPS can act as a powerful adjuvant for T cell responses to specific antigen (19–21). It has also been found that LPS can stimulate certain T cell clones and a small proportion (1–3%) of splenic T cells in vitro (22). However, quantitative information on the capacity of LPS to stimulate normal T cells in vivo has not been reported.

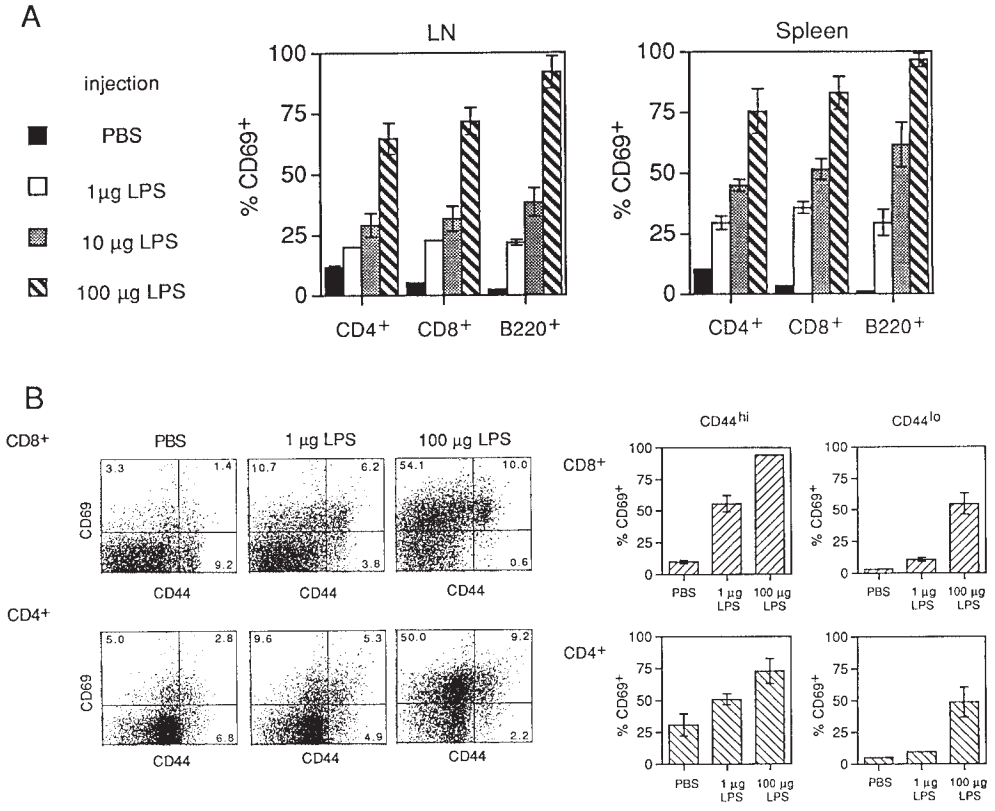

Figure 6. Expression of CD69 on T and B cells after LPS injection. (*A*) CD69 expression on total $CD4^+$, $CD8^+$ and $B220^+$ LN (*left*) and spleen cells (*right*) 1 d after injection of either PBS or 1, 10, or 100 μg LPS. Data represent mean values from two mice per point (± SD). (*B*) CD69 expression on $CD8^+$ (*upper*) or $CD4^+$ (*lower*) LN T cells expressing different levels of CD44 after injection of either PBS or 1 or 100 μg LPS. The percent labeling shown on the right in *B* was derived from an independent experiment from that in *A*, and the data represent mean values from two mice per point. In both experiments, PBS or LPS was injected intravenously into B6 mice, and LN and spleen cells were analyzed 1 d later.

We show here that injecting LPS into normal mice placed on BrdU water induced substantial proliferation of T cells. Whereas proliferation of B cells required quite high doses of LPS (10 μg), even very low doses of LPS (10 ng) were sufficient to cause T cell proliferation. At all doses of LPS tested, the proliferative response of T cells was restricted to a single subset of T cells, namely $CD44^{hi}$ $CD8^+$ cells. However, in terms of CD69 upregulation, LPS injection led to activation of nearly all T cells, including naive ($CD44^{lo}$) $CD8^+$ and $CD4^+$ cells.

The strong proliferation of $CD44^{hi}$ $CD8^+$ cells induced by LPS injection correlated closely with the selective proliferation of $CD44^{hi}$ $CD8^+$ cells seen previously in viral infections and in mice injected with IFN-I or Poly I:C (9). Hence, it is highly likely that LPS-induced proliferation of $CD44^{hi}$ $CD8^+$ cells requires the production of IFN-I, presumably via a direct action of LPS on APC. A critical role for APC is apparent from the finding that the failure of LPS to stimulate T cell proliferation in LPS-nonresponder mice could be overcome by coinjecting normal T-depleted spleen cells as a source of APC. Although LPS can stimulate APC to produce a spectrum of different cytokines (11–13, 23–25), it is notable that T cell stimulation after LPS injection was substantially reduced in IFN-IR^- mice, though only with limiting doses of LPS. Therefore, the implication is that LPS stimulation of $CD8^+$ cells is strongly dependent on IFN-I production but only at moderate or low doses of LPS. At higher LPS doses, IFN-I production is apparently no longer essential, and T cell proliferation is controlled by a different mechanism. Two obvious questions arise:

Does IFN-I act directly on T cells? This possibility is unlikely because we have found no evidence that IFN-I is capable of causing proliferation of purified T cells in vitro (our unpublished data). Moreover, based on studies with bone marrow chimeras constructed with a mixture of normal and IFN-IR^- stem cells, the inability of IFN-IR^- T cells to proliferate after IFN-I (Poly I:C) injection can be overcome by providing a source of normal APC (our unpublished data). These findings suggest that IFN-I induces T cell proliferation by an indirect pathway involving APC. The simplest possibility is that IFN-I stimulates APC to produce other cytokines, which then act directly on T cells.

Which cytokines elicit T cell proliferation in vivo? Because a wide variety of different cytokines are capable of stimulating activated T cells in vitro, defining which particular cytokines stimulate T cells after LPS injection in vivo is a formidable task. IL-12 is a logical candidate because this cytokine is synthesized by APC in response to LPS (13). Moreover, we have observed strong stimulation of $CD44^{hi}$ $CD8^+$ cells in mice injected with rIL-12 (our unpublished data). Nevertheless, we have seen only minimal proliferation of T cells after culture with IL-12 in vitro. Hence, as with IFN-I, it is doubtful whether IL-12 acts directly on T cells. In considering other cytokines, IL-15 is of special interest because this IL-2–like cytokine is produced by APC and, like IL-2, IL-15 is directly stimulatory for activated T cells in vitro (25, 26). Experiments with IL-15 (and various other cytokines) are currently underway.

Because LPS is a major component of gram-negative bacteria, one would expect to see prominent evidence of T cell proliferation during bacterial infections. In fact, as with LPS, we have found that injecting mice with killed *Brucella abortus* causes marked proliferation of $CD44^{hi}$ $CD8^+$ cells (our unpublished data). However, in contrast with LPS, *B. abortus* induces T cell proliferation in both normal and LPS-nonresponder mice, indicating that T cell stimulation by bacteria is not controlled solely by LPS. Hence, bearing in mind that bystander stimulation of $CD44^{hi}$ $CD8^+$ cells is also conspicuous in viral infections (9), it would seem quite likely that many different products of infectious microorganisms have the capacity to stimulate memory-phenotype T cells in vivo. However, in normal animals it is notable that the background rate of proliferation of memory–phenotype T cells is substantially higher for $CD4^+$ cells than for $CD8^+$ cells (3). This is surprising because the stimulation of $CD44^{hi}$ cells induced by viruses, LPS, and killed *B. abortus* is heavily skewed to $CD8^+$ cells. Therefore, the implication is that stimulation of $CD44^{hi}$ $CD4^+$ cells is controlled by a separate mechanism. In this respect, preliminary work has shown that certain bacteria induce poor proliferation of $CD44^{hi}$ $CD8^+$ cells in vivo but strong proliferation of $CD44^{hi}$ $CD4^+$ cells (our unpublished data). Which cytokines control the proliferation of $CD44^{hi}$ $CD4^+$ cells has yet to be resolved.

As a final comment, it is striking that high doses of LPS caused marked CD69 upregulation on $CD44^{lo}$ (naive) cells as well as on $CD44^{hi}$ cells, both for $CD4^+$ cells and $CD8^+$ cells. How LPS induces activation of naive T cells but without causing these cells to enter cell cycle is unknown. Future work will be needed to resolve this paradox.

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