

A Natural Immunological Adjuvant Enhances T Cell Clonal Expansion through a CD28-dependent, Interleukin (IL)-2-independent Mechanism

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Summary

The adoptive transfer of naive CD4⁺ T cell receptor (TCR) transgenic T cells was used to investigate the mechanisms by which the adjuvant lipopolysaccharide (LPS) enhance T cell clonal expansion in vivo. Subcutaneous administration of soluble antigen (Ag) resulted in rapid and transient accumulation of the Ag-specific T cells in the draining lymph nodes (LNs), which was preceded by the production of interleukin (IL)-2. CD28-deficient, Ag-specific T cells produced only small amounts of IL-2 in response to soluble Ag and did not accumulate in the LN to the same extent as wild-type T cells. Injection of Ag and LPS, a natural immunological adjuvant, enhanced IL-2 production and LN accumulation of wild-type, Ag-specific T cells but had no significant effect on CD28-deficient, Ag-specific T cells. Therefore, CD28 is critical for Ag-driven IL-2 production and T cell proliferation in vivo, and is essential for the LPS-mediated enhancement of these events. However, enhancement of IL-2 production could not explain the LPS-dependent increase of T cell accumulation because IL-2-deficient, Ag-specific T cells accumulated to a greater extent in the LN than wild-type T cells in response to Ag plus LPS. These results indicate that adjuvants improve T cell proliferation in vivo via a CD28-dependent signal that can operate in the absence of IL-2.

Clonal expansion of Ag-specific T cells during a primary immune response is critical for the development of subsequent immunological memory (1). However, expansion of Ag-specific T cells after exposure to a soluble foreign Ag is typically only transient and is quickly followed by the deletion and/or functional inactivation of the cells (2, 3). Such abortive immune responses can be rescued by the addition of immunological adjuvants that increase the proliferation of Ag-specific T cells and prolong their survival and functional competence (1–4). Multiple substances have adjuvant properties, but the mechanisms responsible for adjuvant activity are poorly understood.

Because adjuvants increase the number of Ag-specific T cells that accumulate in lymphoid tissues, it is likely that they affect T cell growth factor production. In vitro studies have established that IL-2 is a potent T cell growth factor (5) and that at least two signals are required for optimal IL-2 production: one provided by the interaction of the TCR with peptide–class II MHC complexes, and the second by

binding of costimulatory molecules like CD28 to B7-1 and B7-2, present on APCs (6, 7). Because adjuvants have been shown to induce B7 molecules on APCs (8, 9), it is possible that adjuvants improve the costimulatory functions of APCs, which stimulate T cells to produce more IL-2 and proliferate more extensively. Here, we tested this model by studying the clonal expansion of wild-type, CD28-deficient, or IL-2-deficient TCR transgenic T cells in vivo after adoptive transfer into normal recipients and priming these recipients with Ag plus or minus adjuvant. Our results lead to the surprising conclusion that adjuvants improve T cell clonal expansion via CD28-mediated enhancement of a growth factor other than IL-2. In fact, IL-2 appeared to play an inhibitory role because the long-term persistence of Ag-specific T cells after antigenic challenge in vivo was actually improved in the absence of IL-2.

Materials and Methods

Mice and the Adoptive Transfer Protocol. BALB/c mice were purchased from Sasco (Omaha, NE) or The Jackson Laboratories (Bar Harbor, ME). The DO11.10 TCR transgenic mice (10) were bred in a specific pathogen-free facility according to Na-

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tional Institutes of Health guidelines, and screened for transgene expression as previously described (2). These mice had been extensively backcrossed (>15 generations) onto the BALB/c background and, therefore, are histocompatible with normal BALB/c mice. CD4⁺, KJ1-26⁺ TCR transgenic cells were adoptively transferred (2.5–5 × 10⁶ cells per mouse) by intravenous injection into unirradiated BALB/c mice as previously described (2). CD28-deficient mice (generously provided by Dr. Craig B. Thompson, University of Chicago, IL) were backcrossed to DO11.10 BALB/c mice for five generations. No evidence for rejection of transferred CD28-deficient DO11.10 T cells was seen in any of the experiments (over a time course of eight days). DO11.10 BALB/c mice were crossed for two generations with BALB/c SCID mice obtained from The Jackson Laboratories to produce DO11.10 TCR transgenic BALB/c SCID donors. IL-2^{+/-} BALB/c mice, obtained from The Jackson Laboratories, were crossed for two generations with the DO11.10 mice and screened by PCR for the presence of the targeted IL-2 gene and by flow cytometry for the presence of the DO11.10 TCR. The IL-2-deficient DO11.10 mice were not observed to have overt clinical manifestations of an autoimmune disease described in the BALB/c IL-2-deficient mice (11) for at least 3 mo, although they did develop lymphadenopathy and splenomegaly. Furthermore, the dominant population of CD4⁺, KJ1-26⁺ cells in these mice had the surface phenotype of naive cells, that is, CD45RB^{high}, LFA^{low}, CD69^{low} (data not shown).

Adoptive Transfer of CD45RB^{high} and CD45RB^{low} DO11.10 T Cells. A subpopulation of transgenic DO11.10 T cells expressed a second TCR and were CD45RB^{low} due to previous activation (12). LN and spleen cells were obtained from wild-type and IL-2-deficient DO11.10 mice. Cells were stained and sorted similarly, with some modifications, to a protocol described by Powrie et al. (13). In brief, erythrocytes were removed by hypotonic lysis using ACK Buffer (Biofluids, Inc., Rockville, MD) and CD4⁺ T cells were enriched by negative depletion using magnetic beads (Dyna, Oslo, Norway) coated with anti-B220, anti-CD8, anti-I-A^d, and anti-HSA. The anti-HSA mAb proved critical for efficient enrichment of cells obtained from IL-2-deficient mice because of the greatly expanded population of HSA⁺ cells negative for other markers in these mice (11). Enriched CD4⁺ T cells (75–95% pure) were labeled with FITC-conjugated anti-CD45RB mAb for 15 min on ice and separated into CD45RB^{high} and CD45RB^{low} fractions by single-color sorting on a FACSVantage[®] flow cytometer (Becton Dickinson, Mountain View, CA). Sorted cells were then transferred into syngeneic BALB/c recipients as described above.

Flow Cytometric Analysis of LN Cells. Chicken OVA (Sigma Chemical Co., St. Louis, MO) was injected subcutaneously (typically 2 mg with or without 50–150 µg of LPS; serotype *Escherichia coli* 026:B6; Difco Laboratories, Detroit, MI) into four sites on the back. Axillary, brachial, and inguinal LNs were harvested at various times, and the transferred DO11.10 T cells were identified by two-color flow cytometric analysis as previously described (2). In brief, 2 × 10⁶ LN cells were incubated on ice with PE-labeled anti-CD4 mAb (PharMingen, San Diego, CA) and biotinylated KJ1-26 mAb, which binds exclusively to the DO11.10 TCR. Cells were then washed and incubated with FITC-labeled streptavidin (SA; Caltag, South San Francisco, CA) to detect the KJ1-26 mAb. 20,000 events were then collected for each sample on a FACScan[®] flow cytometer and analyzed using Lysis II software. DO11.10 T cells were identified as CD4⁺, KJ1-26⁺ cells. The total number of DO11.10 T cells present was calculated by multiplying the total number of viable LN cells (obtained by counting viable cells) by the percentage of CD4⁺, KJ1-26⁺ cells obtained by flow cytometry.

Reverse Transcription PCR. Total RNA was isolated from LN cell suspensions according to the RNAzol B procedure (Tel-Test, Inc., Friendswood, TX). In some cases, the LN cells were depleted of DO11.10 cells using the KJ1-26 mAb and magnetic beads according to the manufacturer's protocol. 2 µg of total RNA were used in an oligo-dT-mediated reverse transcription (RT)-PCR in a total volume of 20 µl. One-tenth of the resulting cDNA was amplified by PCR as previously described with sense and antisense primers derived from the *IL-2* gene or the *hypoxanthine-guanine phosphoribosyl transferase (HPRT)*¹ gene, which was used as internal control (14). PCR products were then separated on 1% agarose gels, transferred to nylon membranes, detected by conventional Southern blot techniques with ³²P-labeled *IL-2* and *HPRT* cDNA probes, and then visualized by autoradiography. In some experiments, the cDNAs were amplified with the *IL-2* and *HPRT* primer sets in the same reaction in the presence of α-[³²P]dCTP (0.2 µCi/tube). The PCR products were then separated on 5% urea-polyacrylamide gels, visualized by autoradiography, and then analyzed by densitometry. Because the PCR reactions were still in the linear range for both *HPRT* and *IL-2* products (23 cycles), the optical units (OU) obtained for the *HPRT* bands were used as an internal standard for the total amount of RNA/cDNA used in each reaction. The amount of OVA-induced *IL-2* mRNA was then calculated according to the following formula: [OU (*IL-2*) / OU (*HPRT*)]_{injected} – [OU (*IL-2*) / OU (*HPRT*)]_{uninjected}. Since *IL-2* mRNA produced by endogenous OVA-specific T cells cannot be detected by this technique (Mondino, A., unpublished observations) all of the OVA-induced *IL-2* mRNA measured must be derived from the transferred DO11.10 T cells.

IL-2 Protein Intracellular Staining. A staining protocol based on work of others (15) was used to detect *IL-2* production by transferred DO11.10 T cells. LN cells (3 × 10⁶) isolated at various times after Ag injection were stained with anti-CD4 Cy-Chrome and biotinylated KJ1-26, followed by SA-FITC in staining buffer (PBS plus 2% fetal calf serum and 0.2% sodium azide). The cells were then washed with PBS, fixed for 20 min at room temperature in PBS containing 2% formaldehyde, permeabilized with two washes in staining buffer containing 0.5% saponin (Sigma Chemical Co.), and then incubated for 30 min at room temperature with PE-labeled anti-*IL-2* mAb, or with a PE-labeled irrelevant mAb of the same isotype (PharMingen). The cells were then washed once with saponin buffer and twice with PBS, and at least 1,000 CD4⁺, KJ1-26⁺ events (as well as an equal number of CD4⁺, KJ1-26⁻ events) were collected for each sample on a FACScan[®] flow cytometer. For most experiments the amount of intracellular *IL-2* protein present after OVA injection was calculated as the percentage increase in the mean fluorescence intensity (MFI) of *IL-2*-stained CD4⁺, KJ1-26⁺ cells according to the following formula: [(MFI)_{injected} – (MFI)_{uninjected}] / (MFI)_{uninjected} × 100.

It should be noted that LN cells were stained immediately after isolation. No additional incubation in the presence of Brefeldin A, an inhibitor of exocytosis important for detection of intracellular cytokines produced in response to *in vitro* stimulation (15), was required since this compound did not enhance the amount of intracellular *IL-2* detected in freshly explanted LN cells. However, Brefeldin A did prevent the loss of intracellular *IL-2* that

¹Abbreviations used in this paper: γ_c, common γ chain; *HPRT*, hypoxanthine-guanine phosphoribosyl transferase; MFI, mean fluorescence intensity; OU, optical units; RT, reverse transcription; SA, streptavidin.

occurred when LN cells were incubated in vitro at 37°C for an additional 2–3 h (Khoruts, A., unpublished observations).

ELISA Measurement of IL-2 Production by DO11.10 T Cells After In Vivo OVA Stimulation. LN cells ($3\text{--}5 \times 10^6$) from OVA-injected or uninjected adoptive transfer recipients were suspended in 0.2 ml of complete medium (Eagle's Hanks' amino acids medium; Biofluids) supplemented with 10% FCS, penicillin G (100 U/ml), streptomycin (100 $\mu\text{g}/\text{ml}$), gentamicin (20 $\mu\text{g}/\text{ml}$), L-glutamine (2×10^{-3} M), and 2-mercaptoethanol (5×10^{-5} M), and incubated in vitro at 37°C without additional Ag for 2–4 h. IL-2 secreted into the supernatant was measured by sandwich ELISA based on noncompeting pairs of anti-IL-2 mAbs, JES6-1A12 and JES6-5H4 (PharMingen) according to the protocol provided by the manufacturer. IL-2 was not detected in the culture supernatants of LN cells from Ag-primed animals that did not receive DO11.10 T cells (Khoruts, A., and A. Mondino, unpublished observation). Since the IL-2 signal was completely dependent on the presence of DO11.10 T cells, the mean amount of IL-2 secreted by individual DO11.10 cells was calculated by dividing the amount of IL-2 produced in the culture by the number of CD4⁺, KJ1-26⁺ cells present.

Measurement of IL-2, IL-3, IL-5, and IFN- γ Production after In Vitro Ag Stimulation Using ELISA. LN cells (2.5×10^6) from OVA/LPS-injected or uninjected adoptive transfer recipients were suspended in 0.25 ml of complete medium and incubated in the presence or absence of 10 μM OVA peptide 323–339 for 48 h. Cytokines present in the culture medium were measured by sandwich ELISA based on noncompeting pairs of anti-IL-2 (JES6-1A12 and JES6-5H4), anti-IL-3 (MP2-8F8 and MP2-43D11), anti-IL-5 (TRFK5 and TRFK4), or anti-IFN- γ mAbs (R4-6A2 and XMG1.2; PharMingen) according to the protocol provided by the manufacturer. IL-2 could not be detected after stimulation of IL-2-deficient DO11.10 T cells, and cytokine production was dependent on addition of the OVA peptide. Amounts of cytokine produced were either calculated based upon the standard curve generated by known amounts of cytokine, or expressed as relative units based upon OD values. All cytokine concentrations were then adjusted for the number of CD4⁺, KJ1-26⁺ cells present at the start of the culture period.

In Vivo Ab Treatments. Rat mAbs specific for murine B7-1 (1G10) or B7-2 (GL-1) were generously provided by Dr. Bruce Blazar (University of Minnesota, Minneapolis, MN). Anti-B7 or rat IgG control antibodies were injected intravenously into mice 6 h before Ag administration and intraperitoneally at the time of Ag administration (75 $\mu\text{g}/\text{injection}$). CTLA-4-Ig fusion protein (kindly provided by Dr. Robert Karr, Monsanto Company, St. Louis, MO) was purified over protein A-agarose. CTLA-4-Ig was injected intravenously (100 $\mu\text{g}/\text{injection}$) 6 h before Ag administration and intraperitoneally at the time of Ag administration. Neutralizing anti-IL-2 mAb S4B6 or rat IgG control antibodies were injected 2 h before (1 mg) and 8, 24, and 48 h (0.5 mg) after Ag administration.

Results

Quantitative Detection of IL-2 mRNA and Protein In Vivo after Injection of Soluble OVA. The adoptive transfer system used here allowed physical tracking of Ag-specific CD4⁺ T cells during OVA-induced immune responses in vivo (2). CD4⁺ T cells obtained from OVA peptide/I-A^d-specific, DO11.10 TCR transgenic mice were transferred into otherwise unmanipulated syngeneic BALB/c recipi-

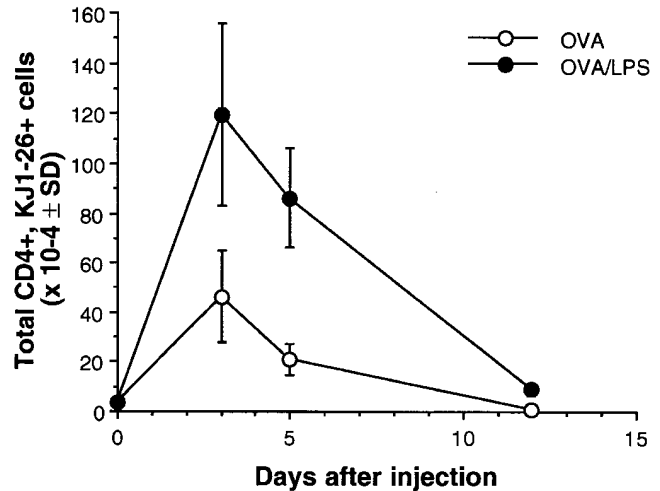


Figure 1. LPS increases CD4⁺, KJ1-26⁺ clonal expansion. The total number of CD4⁺, KJ1-26⁺ cells was measured in the draining LNs (axillary, brachial, inguinal, and cervical) after s.c. injection of 2 mg OVA without (open circles) or with LPS (filled circles). One representative experiment of 15 is shown.

ents. The DO11.10 T cells were detected with the anticlonotypic mAb, KJ1-26, by flow cytometry (2). Clonal expansion of transferred DO11.10 T cells in response to soluble Ag injection was greatly enhanced by immunological adjuvants such as CFA (2) or LPS (Fig. 1 and reference 4). This increase was apparent at all time points examined including the time of peak DO11.10 T cell accumulation on day 3, and on days when the number of DO11.10 T cells had decreased slightly (day 5) or dramatically (day 12).

A mechanism by which adjuvants could affect T cell clonal expansion is enhancement of growth factor production. IL-2 was studied because it is the major T cell growth factor produced by naive CD4⁺ T cells after stimulation in vitro (16), and IL-2-deficient CD4⁺ T cells proliferate less well than normal CD4⁺ T cells after activation (17–19).

Production of IL-2 mRNA by the DO11.10 T cells was investigated using RT-PCR (Fig. 2 A). Injection of soluble OVA resulted in a marked increase in IL-2 mRNA expression in the draining LN cells of adoptive transfer recipients (Fig. 2 A, LN Cells). The OVA-induced IL-2 mRNA was exclusively expressed by the DO11.10 T cells because the inducible signal was lost whenever RNA was isolated from LN cells depleted of DO11.10 T cells with KJ1-26-coated magnetic beads (Fig. 2 A, KJ1-26 Depleted) and retained when RNA was isolated from the cells that adhered to the KJ1-26-coated beads (Fig. 2 A, Beads).

Intracellular staining was used to detect IL-2 protein (15, 20). IL-2 protein was detected in a subset of CD4⁺, KJ1-26⁺ T cells (identified as shown in Fig. 2 B) recovered from mice injected subcutaneously 10 h before with OVA (Fig. 2 C, histogram 3). No staining was detected in CD4⁺, KJ1-26⁻ T cells present in the same tube (Fig. 2 C, histogram 2), in CD4⁺, KJ1-26⁺ T cells stained with an isotype-matched control mAb (Fig. 2 C, histogram 1), or in CD4⁺,

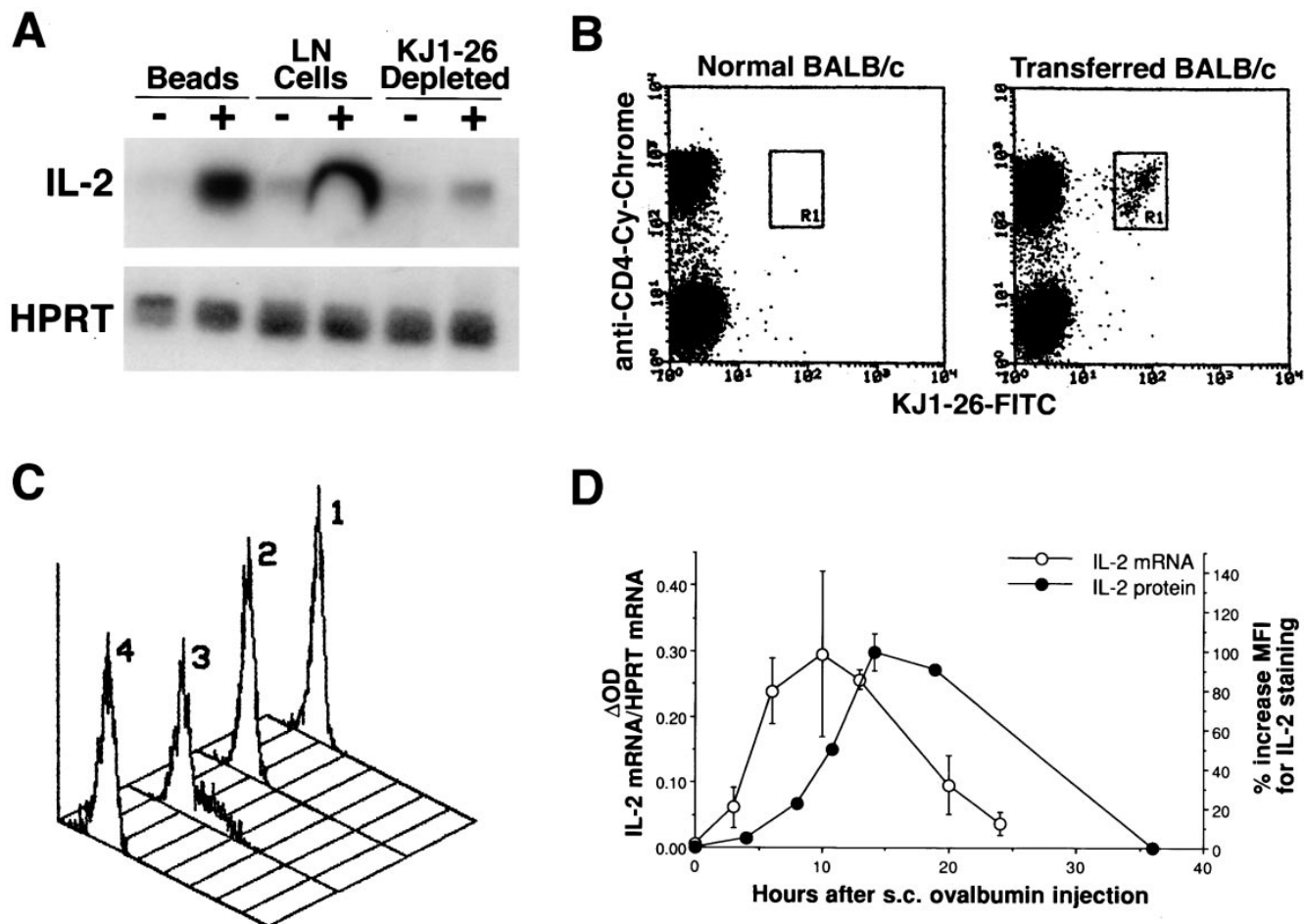


Figure 2. Ag-specific CD4⁺ T cells express IL-2 mRNA and protein after Ag injection. (A) Adoptively transferred mice were either uninjected (-) or injected subcutaneously with soluble OVA (+). Peripheral LNs were harvested 6 h after Ag injection. Total RNA was obtained from unfractionated LN cells (LN Cells), from cells that were depleted of DO11.10 T cells by KJ1-26 mAb-coated magnetic beads (KJ1-26 depleted), and from magnetic bead-enriched KJ1-26⁺ cells (Beads). IL-2 and HPRT RT-PCR products were detected by Southern blot and autoradiographic techniques. (B) BALB/c mice were injected with 2.5×10^6 DO11.10 T cells as described in the Materials and Methods section. FACS[®] profiles for brachial, axillary, and inguinal LN cells from normal (left) or adoptive transfer mice (right) stained with Cy-Chrome-labeled anti-CD4 mAb and FITC-labeled KJ1-26 mAb are shown. (C) Cells in B, right were further stained with PE-labeled anti-IL-2 mAb. Histograms represent PE-channel fluorescence of stained LN cells recovered from uninjected (histogram 2) or OVA-injected animals (histograms 1, 3, and 4). Histogram 1, CD4⁺, KJ1-26⁺ cells stained with PE-labeled isotype matched mAb (rat IgG2a); histogram 2, CD4⁺, KJ1-26⁺ cells from an uninjected mouse stained with PE-labeled anti-IL-2 mAb; histograms 3 and 4, CD4⁺, KJ1-26⁺ and CD4⁺, KJ1-26⁻ cells stained with PE-labeled anti-IL-2 mAb. (D) The kinetics of IL-2 mRNA (open circles) and protein (filled circles) expression after s.c. injection of 2 mg OVA. Data from three separate experiments on the kinetics of IL-2 mRNA expression were pooled. IL-2 mRNA was detected by RT-PCR and quantified as described in Materials and Methods. Each point represents the mean from 4–10 mice \pm SEM. IL-2 protein expression was measured as described in B and C. One representative experiment of four is shown. Each point represents the mean of two mice \pm range.

KJ1-26⁺ T cells recovered from animals that received no Ag and were stained with anti-IL-2 mAb (Fig. 2 C, histogram 4). Analysis of OVA-induced IL-2 production over time by DO11.10 T cells indicated that IL-2 mRNA expression peaked 10 h after Ag administration and was barely detectable by 24 h (Fig. 2 D). Very similar results were reported by Rogers et al. (21), who used this adoptive transfer system to detect IL-2 mRNA in situ. As expected, IL-2 protein production lagged behind mRNA production, with peak expression occurring 12–14 h after OVA injection and returning to background levels after 24 h (Fig. 2 D). These results indicate that IL-2 production by OVA-specific T cells after Ag encounter in vivo occurs in a very rapid and tran-

sient fashion. Because the transferred DO11.10 T cells contain a subpopulation of cells (10–30% of the transgenic cells) with a memory phenotype presumably due to activation through a second TCR (12), we repeated the kinetics experiments using adoptively transferred DO11.10 SCID T cells, which are all of naive phenotype. The kinetics of IL-2 production by these cells was indistinguishable from that of wild-type DO11.10 T cells (data not shown), thereby excluding the possibility of memory cells contributing significantly to the earliest production of IL-2 in this system.

IL-2 Production In Vivo Is Enhanced by LPS and Is Dependent on B7-CD28. Injection of soluble Ag with LPS resulted in increased IL-2 production by the Ag-specific cells.

Table 1. Pooled Data on IL-2 Production by CD4⁺, KJ1-26⁺ Cells In Vivo in Response to Ovalbumin ± LPS

Treatment group	Mice per group	Lane	Parameter of IL-2 staining of CD4 ⁺ , KJ1-26 ⁺ cells	No Antigen	OVA	OVA/LPS	OVA versus OVA/LPS
Control mice: Rat IgG treated or untreated	<i>n</i> = 39	A	Percentage of IL-2 ⁺ cells	<i>mean</i> ± <i>SE</i> 0.22 ± 0.03	<i>mean</i> ± <i>SE</i> 10.7 ± 0.6	<i>mean</i> ± <i>SE</i> 22.6 ± 1.0	<i>P</i> < 0.0001
		B	MFI	NA	32.5 ± 1.0	40.4 ± 1.3	<i>P</i> < 0.0001
		C	Percentage of increase in MFI	NA	60.2 ± 6.4	146.0 ± 12.7	<i>P</i> < 0.0001
CTLA-4-Ig treated mice	<i>n</i> = 7	D	Percentage of IL-2 ⁺ Cells	0.22 ± 0.03	1.3 ± 0.1	2.5 ± 0.6	<i>P</i> = 0.09
		E	MFI	NA	27.3 ± 0.8	29.2 ± 0.5	<i>P</i> = 0.07
		F	Percentage of increase in MFI	NA	13.6 ± 1.8	16.7 ± 2.4	<i>P</i> = 0.39
CD28-deficient DO11.10 T cells	<i>n</i> = 13	G	Percentage of IL-2 ⁺ Cells	0.18 ± 0.04	1.5 ± 0.1	1.5 ± 0.2	<i>P</i> = 0.78
		H	MFI	NA	26.4 ± 1.8	25.3 ± 1.5	<i>P</i> = 0.13
		J	Percentage of increase in MFI	NA	8.5 ± 1.6	10.9 ± 2.7	<i>P</i> = 0.17

Flow cytometric measurements of IL-2 production in vivo by Ag-specific T cells in response to Ag stimulation in the presence or absence of CD28 costimulation. OVA was administered with or without LPS to mice 10–12 h before killing. Lanes A, D, and G show the percent of CD4⁺, KJ1-26⁺ cells that were IL-2⁺. Lanes B, E, H show the MFI for IL-2 staining of only the CD4⁺, KJ1-26⁺, IL-2⁺ cells. Lanes C, F, and J show the percent increase in MFI for IL-2 staining of the total population of CD4⁺, KJ1-26⁺ cells derived from LNs of injected animals as compared to background staining of CD4⁺, KJ1-26⁺ cells from uninjected animals. Lanes A, B, and C show pooled data from 12 independent experiments where mice were transferred with wild-type DO11.10 T cells and were either treated with rat IgG Ab used as a control for CTLA-4-Ig, or were untreated. Lanes D, E, and F show pooled data from three independent experiments where mice were transferred with wild-type DO11.10 T cells and were treated with CTLA-4-Ig. Lanes G, H, and J show pooled data from four independent experiments where mice were transferred with CD28-deficient DO11.10 T cells. Treatment of these mice with CTLA-4-Ig did not further decrease IL-2 production (data not shown). The significance of differences in IL-2 production by DO11.10 T cells from mice injected with OVA alone and OVA plus LPS was tested with the paired two-tailed Student's *t* test.

The effect of LPS was attributable to an increased number of DO11.10 T cells that produced IL-2 (Table 1, lane A) and to greater IL-2 production per DO11.10 T cell (Table 1, lane B and Fig. 3). A similar adjuvant effect was seen when DO11.10 SCID T cells were used in the adoptive transfer (data not shown) demonstrating that naive T cells are the predominant adjuvant-responsive population. The two- to threefold difference in the relative increase in mean fluorescence intensity of IL-2 staining correlated with the differences in IL-2 detected by ELISA in the supernatants of LN cells cultured in vitro in the absence of additional antigenic stimulation (Fig. 3).

It has been proposed that adjuvants enhance T cell responses by increasing the expression of costimulatory molecules on APC (6, 8). If LPS works by this mechanism, then its adjuvanticity should be diminished in the absence of CD28-mediated costimulation. This prediction was tested using adoptive transfer of CD28-deficient DO11.10 T cells. As shown in Fig. 4, CD28-deficient DO11.10 T cells produced small, but significant, amounts of IL-2 in vivo in response to OVA when compared with wild-type DO11.10

T cells. The CD28-deficient DO11.10 T cells were defective at producing IL-2 at each time point tested over the first 20 h after Ag injection. The CD28-deficient DO11.10 T cells did become blasts (Fig. 4 C), however, demonstrating that these cells were stimulated by Ag-bearing APCs in vivo. LPS did not significantly enhance the amount of IL-2 produced by CD28-deficient DO11.10 T cells in response to OVA (Fig. 4, A and B, Table 1). Similarly, when B7 molecules were blocked in vivo with CTLA-4-Ig (Table 1) or a combination of anti-B7-1 and anti-B7-2 mAbs (data not shown), OVA-induced IL-2 production by DO11.10 T cells was dramatically reduced regardless of the presence of adjuvant (Table 1). These results indicate that LPS enhances Ag-driven IL-2 production through a B7/CD28-dependent signal.

The lack of an adjuvant effect of LPS on IL-2 production by CD28-deficient DO11.10 T cells correlated well with the lack of an adjuvant effect of LPS on the expansion of these cells (Fig. 4 D). Interestingly, CD28-deficient DO11.10 T cells retained substantial ability to expand in response to soluble Ag in the absence of adjuvant. Since a

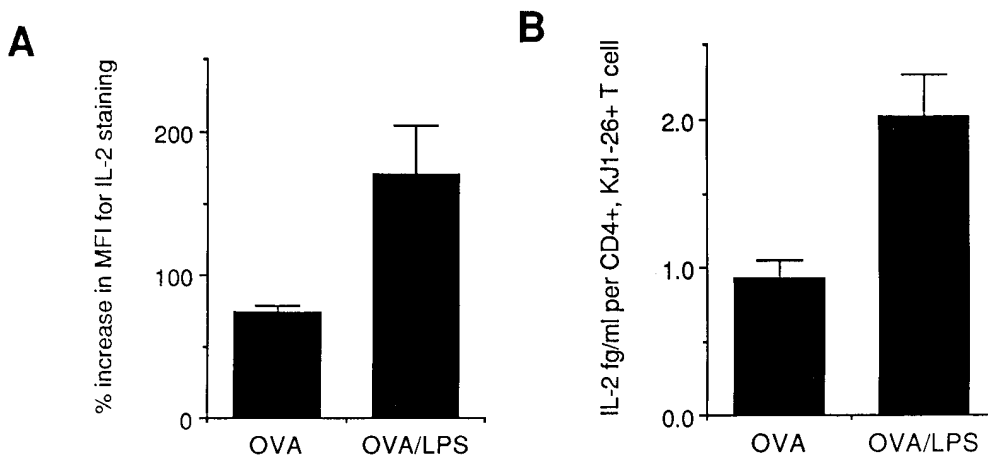


Figure 3. Effect of LPS on IL-2 protein production by Ag-specific T cells. 2 mg of OVA were injected without (OVA) or with LPS (OVA/LPS) 10 h before killing. LN cells were stained for IL-2 directly (A) or incubated in vitro for 2.5 h (B). (A) Intracellular IL-2 protein was measured in the CD4⁺, KJ1-26⁺ population by three-color flow cytometry as described in Fig. 2, B–D. (B) IL-2 protein secreted into the supernatants during the ex vivo culture was measured by ELISA. Each column represents two mice and is shown as the mean \pm range. No IL-2 could be detected in the culture supernatants of LN cells recovered from animals that were transferred with DO11.10 TCR transgenic T cells, but did not receive OVA. The figure is representative of two independent experiments.

number of other costimulatory molecules have been described (22), it is reasonable to suggest that the residual T cell proliferation seen in the absence of B7-CD28 signaling is driven by alternative costimulatory pathways.

IL-2 Is a Critical T Cell Growth Factor In Vitro, but Is Not Absolutely Required In Vivo. Multiple in vitro studies have identified IL-2 as a major T cell growth factor (5) and previous reports have shown that CD4⁺ T cells from IL-2-deficient mice proliferate less well in vitro than wild-type CD4⁺ T cells (17–19). Because of this and our finding that adjuvants enhance in vivo IL-2 production by Ag-specific T cells, it was of interest to determine whether IL-2 was critical for the differences in clonal expansion observed when Ag was injected alone or with adjuvant. As expected, we found that IL-2-deficient, DO11.10 T cells proliferated poorly in response to in vitro stimulation with OVA plus APCs when compared with wild-type DO11.10 T cells (Fig. 5 A). However, when IL-2-deficient DO11.10 T cells were adoptively transferred into normal recipients, they accumulated in the draining LNs to the same extent as wild-type cells on day 3 after OVA injection (Fig. 5 B). Surprisingly, injection of OVA with LPS, a condition where wild-type cells produce more IL-2 (Fig. 4 B), caused the IL-2-deficient DO11.10 T cells to accumulate in draining LNs to a significantly higher level than wild-type cells (Fig. 5 B).

Although these results suggested that IL-2 was not required for maximal clonal expansion of naive T cells, it was formally possible that the IL-2-deficient DO11.10 T cells used for transfer were enriched for memory T cells that use a growth factor other than IL-2. DO11.10 T cells from IL-2-deficient donors contained more memory phenotype cells (~40%) than DO11.10 T cells from normal donors (10–30%). However, as shown in Fig. 6, FACS[®]-sorted, CD45RB^{high} DO11.10 T cells from IL-2-deficient or normal donors underwent a similar amount of clonal expansion when transferred into normal recipients that were in-

jected with OVA alone, and responses by both populations were enhanced by LPS. In fact, the phenotypically naive populations clonally expanded to a greater degree than their memory phenotype (CD45RB^{low}) counterparts.

A trivial explanation for the lack of a defect in clonal expansion in vivo could have been that the IL-2-deficient, DO11.10 T cells responded to IL-2 produced by the recipient's T cells. To address this possibility, LN accumulation of wild-type DO11.10 T cells in response to OVA administration with or without LPS in the presence of a neutralizing anti-IL-2 mAb was measured. As shown in Fig. 7, DO11.10 T cells in mice injected with OVA and treated with anti-IL-2 mAb or control rat IgG achieved identical levels of LN accumulation on day 3, suggesting that IL-2 is not absolutely required in vivo for the initial phase of Ag-driven T cell clonal expansion. Moreover, on day 6, more DO11.10 T cells remained in the LNs of the anti-IL-2 mAb-treated recipients than in the rat IgG-treated recipients (Figs. 7 and 8), and this difference was particularly dramatic when OVA was injected with LPS (Fig. 8). Therefore, based on experiments with IL-2-deficient T cells and wild-type T cells blocked with anti-IL-2 mAb, we conclude that IL-2 does not have an obligatory role in the initial phase of T cell clonal expansion and may in fact have an inhibitory effect on the long-term persistence of activated T cells.

IL-2 Sensitizes Ag-specific T Cells for Cell Death after Ag Stimulation In Vivo. Previous studies have shown that IL-2R signaling is required for activated T cells to become sensitive to Fas-mediated killing (19, 23). Disruption of such a death pathway could explain why IL-2-deficient, Ag-specific T cells (Fig. 5 A) or wild-type T cells activated in the presence of anti-IL-2 mAb (Fig. 7) persisted longer in vivo after Ag stimulation. Because previous attempts to identify apoptotic DO11.10 T cells in vivo were unsuccessful (2), probably as a result of efficient disposal of apoptotic cells by macrophages, we tested the ability of in vivo-

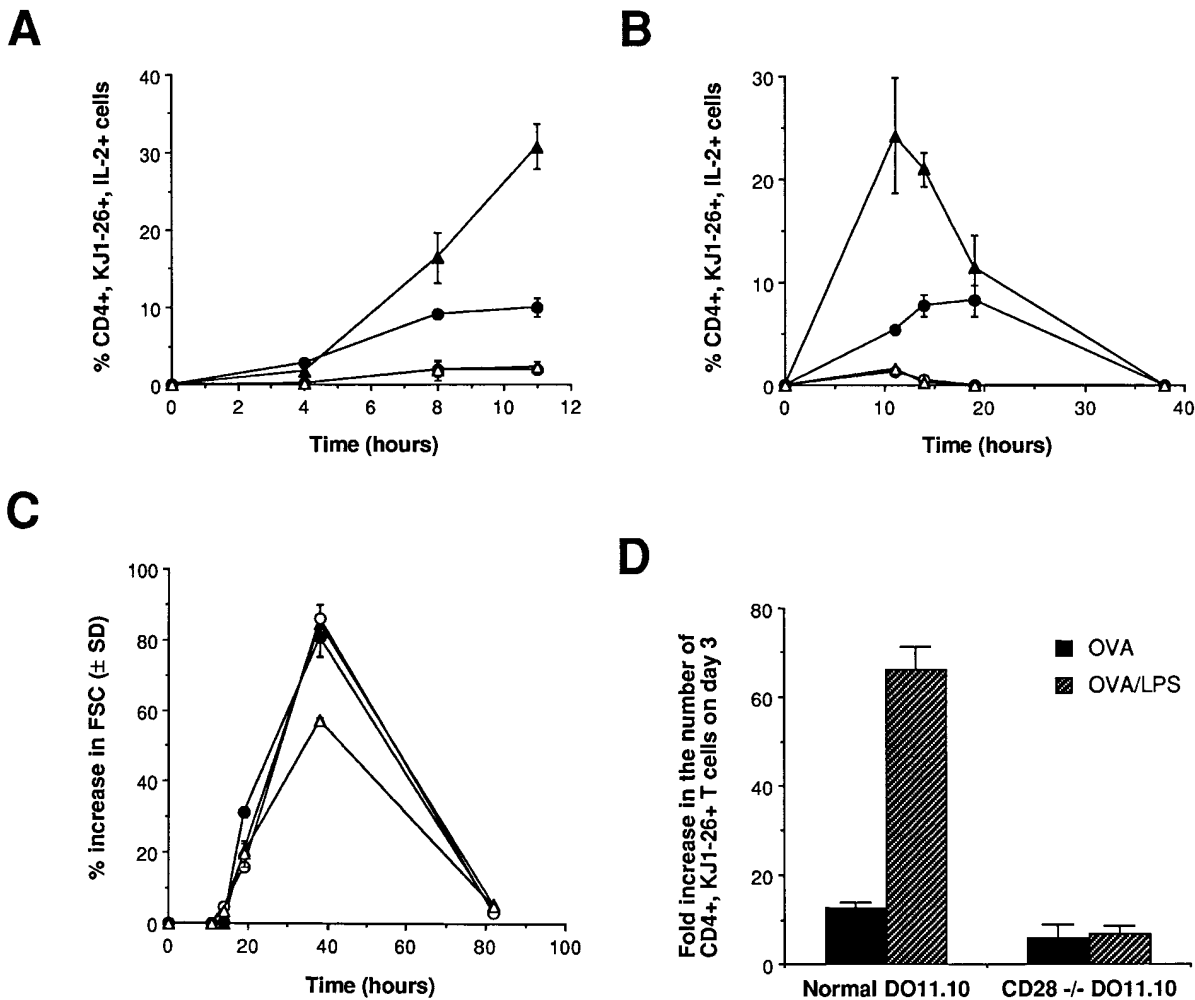


Figure 4. Kinetics of blastogenesis of Ag-specific T cells is normal in the absence of CD28 costimulation, but IL-2 production and Ag-specific T cell expansion are decreased and are not potentiated by LPS. Mice were adoptively transferred with wild-type (*filled symbols*) or CD28-deficient (*open symbols*) DO11.10 cells and injected subcutaneously at different times with OVA with (*triangles*) or without LPS (*diamonds*). Data shown in *B–D* were obtained from one experiment. Data shown in panel *A* covering earlier time points were from a separate experiment. *C* shows the kinetics of blastogenesis as determined by the percent increase in forward light scatter (size) of CD4⁺, KJ1-26⁺ cells from injected mice as compared with CD4⁺, KJ1-26⁺ cells from uninjected mice. Each point in *A–C* represents the mean \pm SD for 2–4 mice per group. *D* shows the fold increase in the number of Ag-specific T cells on day 3 after Ag injection, calculated as a ratio of the total number of Ag-specific T cells present in the draining LNs from Ag-injected animals divided by the total number of Ag-specific T cells present in the same LNs in uninjected animals. Each column represents the mean of data obtained from four mice \pm SD. This experiment is representative of four independent experiments.

activated T cells to survive in culture to determine whether IL-2 plays a role in activation-induced cell death in this system. LN cells, recovered from adoptively transferred mice injected 3 d previously with OVA, were cultured for an additional 3 d in the absence of any additional stimuli. While in vivo Ag-activated, wild-type DO11.10 TCR transgenic T cells died quickly in vitro, the IL-2-deficient DO11.10 TCR transgenic T cells persisted as well as unactivated T cells (Fig 5 *C* and data not shown). These results suggest that death signals dependent on the presence of IL-2 may be received early after Ag activation in vivo.

Functional Response of IL-2-deficient DO11.10 T Cells after Immunization with OVA and LPS. Because LPS had a greater effect on clonal expansion of IL-2-deficient DO11.10 T cells when compared with wild-type DO11.10 cells, it was of

interest to determine if IL-2 may play a role in the differentiation of naive Ag-specific T cells during a primary immune response. Mice were adoptively transferred with naive, IL-2-sufficient DO11.10 SCID T cells or FACS[®]-sorted CD45RB^{high}, IL-2-deficient DO11.10 T cells and left untreated or were immunized with OVA and LPS. On day 8 after immunization cells from the draining LNs were stimulated with the OVA peptide in vitro and cytokines were measured in the culture supernatants (Table 2). Naive, IL-2-sufficient DO11.10 T cells produced IL-2 and IL-3, but very little IFN- γ or IL-5, lymphokines produced by differentiated T cells. IL-2-sufficient DO11.10 T cells from immunized mice produced less IL-2 and IL-3 and detectable amounts of IFN- γ and IL-5, indicating that the DO11.10 T cells had differentiated in vivo. IL-2 was not detected in

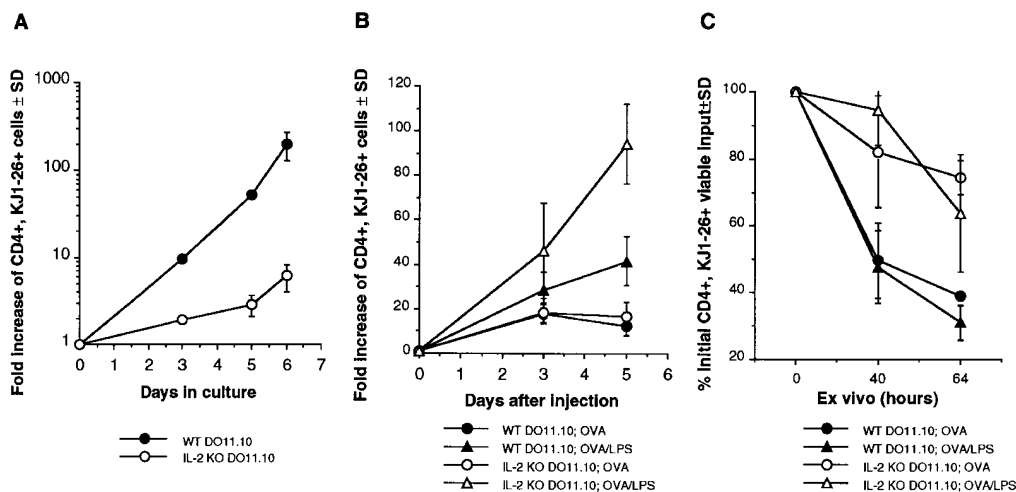


Figure 5. OVA-specific IL-2-deficient T cells have defective proliferative responses in vitro, but not in vivo, and are resistant to cell death. (A) Cells recovered from mice adoptively transferred with wild-type (filled circles) or IL-2-deficient (open circles) DO11.10 T cells were incubated in vitro in the presence of 0.5 μ M OVA peptide 323-339, for the indicated number of days. At each time point replicate wells were harvested and the cells were counted and stained for CD4 and KJ1-26. Mean fold increase \pm SD of the total number of transgenic T cells is shown. (B) Mice adoptively transferred with wild-type (open symbols) or IL-2-deficient (filled symbols) DO11.10 T cells were immunized with OVA in the absence or in the presence of 150 μ g of LPS. The total number of CD4⁺, KJ1-26⁺ T cells was assessed as described in Fig. 1. Each point on the graph represents the mean \pm SE for five mice per group. (C) LN cells recovered from mice adoptively transferred with wild-type (open symbols) or IL-2-deficient (filled symbols) DO11.10 T cells that were injected in vivo with either OVA (circles) or with OVA/LPS (triangles) 3 d before the start of ex vivo culture in the absence of any additional Ag stimulation. The cells were stained at the indicated times for CD4 and KJ1-26 and analyzed by flow cytometry. The mean percentage of initial viable input \pm SD of two animals per group is shown.

the cultures containing naive IL-2-deficient DO11.10 cells, indicating that endogenous OVA-specific T cells did not contribute significantly to cytokine production at this time point. Very little IFN- γ was seen in the cultures containing IL-2-deficient DO11.10 T cells from immunized mice, and IL-3 production was similar to that of naive DO11.10 T cells. In addition, the failure to produce large amounts of IFN- γ was not accompanied by significant skewing toward a Th2 phenotype, as comparable amounts of IL-5 were produced by IL-2-deficient and IL-2-sufficient DO11.10

cells from immunized mice. Therefore, unlike IL-2-sufficient T cells, IL-2-deficient T cells did not differentiate into IFN- γ -producing T cells in vivo in response to immunization.

Discussion

We tested the idea that immunological adjuvants, such as LPS, enhance Ag-induced T cell clonal expansion by improving CD28 signaling and IL-2 production by Ag-acti-

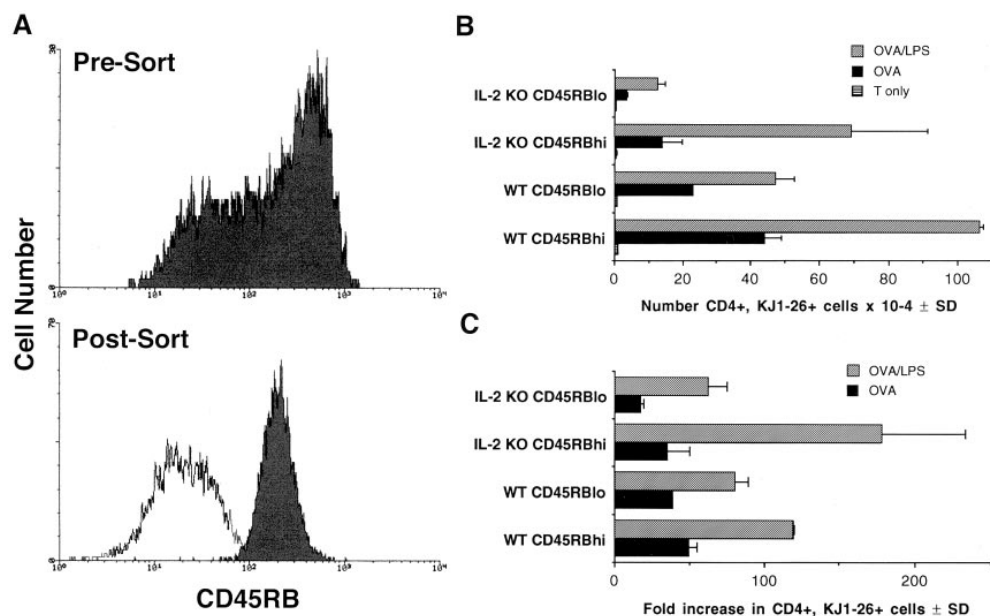


Figure 6. LPS enhances clonal expansion of naive and memory Ag-specific T cells with and without IL-2 production. CD4⁺ T cells from wild-type or IL-2-deficient DO11.10 transgenic mice were sorted into CD45RB^{high} and CD45RB^{low} cells and injected into normal BALB/c mice. The recipients were injected with 2 mg of OVA and 150 μ g of LPS, or 2 mg of OVA alone, or left uninjected. (A) The histogram in the upper panel shows CD45RB staining of CD4⁺ T cells from the IL-2-deficient DO11.10 transgenic donor before sorting. The histograms in the lower panel show equal numbers of sorted CD45RB^{high} and CD45RB^{low} IL-2-deficient CD4⁺ T cells just before the adoptive transfer. (B) Total numbers of CD4⁺, KJ1-26⁺ cells present in the draining LNs on day 3 after Ag injection are shown. Each column represents the mean \pm SD with two mice per group. Since both CD45RB^{high} and CD45RB^{low} cells were obtained from the same sort and were transferred into the same number of recipients, this figure also shows the relative contributions of naive and memory T cells to clonal expansion in the unsorted adoptive transfer experiments. (C) As the baseline numbers of CD4⁺, KJ1-26⁺ T cells in the LNs of uninjected mice were different for each group, clonal expansion was also represented as fold increase in CD4⁺, KJ1-26⁺ cells. The figure represents one of two experiments with similar results.

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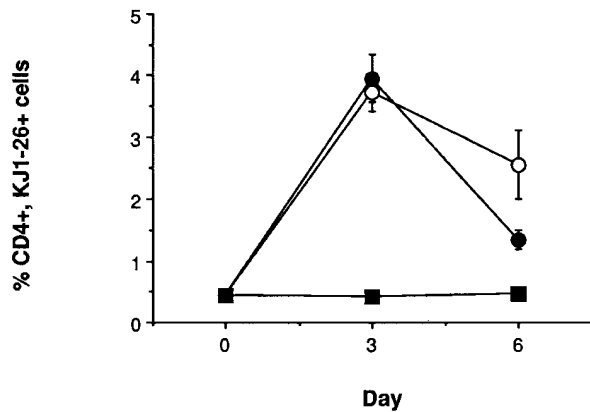
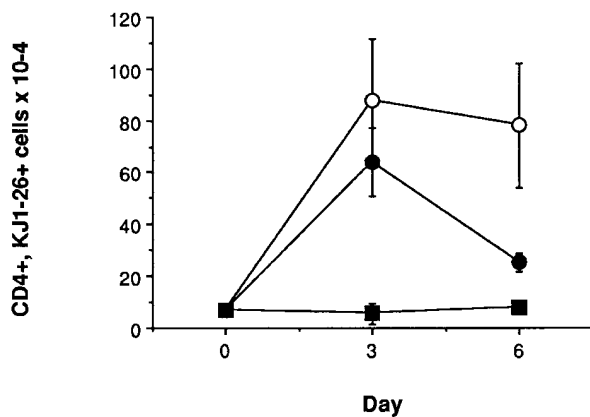
A**B**

Figure 7. In vivo neutralization of IL-2 does not affect T cell clonal expansion and increases T cell persistence. Mice adoptively transferred with wild-type DO11.10 T cells were treated with anti-IL-2 mAb S4B6 or rat IgG as described in Materials and Methods and injected subcutaneously with 2 mg of soluble OVA. The number of CD4⁺, KJ1-26⁺ cells present in the draining LNs was determined 3 and 6 d after Ag injection. Each point represents the mean \pm SE for five mice per group. The anti-IL-2 mAb treatment did not affect the numbers of CD4⁺, KJ1-26⁺ T cells on day 3 in two other independent experiments. (Filled circles) OVA s.c. + rat IgG; (open circles) OVA s.c. + anti-IL-2; (filled squares) T only.

vated T cells in vivo. Although LPS clearly enhanced IL-2 production and clonal expansion via CD28-dependent mechanisms, surprisingly, we found no evidence that IL-2 accounted for the improvement in clonal expansion.

The fact that LPS only enhanced Ag-driven IL-2 production and clonal expansion when CD28 was available for B7 binding is consistent with the idea that the adjuvant effects of LPS are caused by B7 upregulation on APCs. We have recently observed direct interactions between dendritic cells and Ag-specific T cells in the T cell areas of LNs after s.c. OVA injection at a time point that coincides with peak IL-2 production by the T cells (24). Previous studies have shown that the dendritic cells that reside in resting lymphoid tissues express no B7-1 and low levels of B7-2

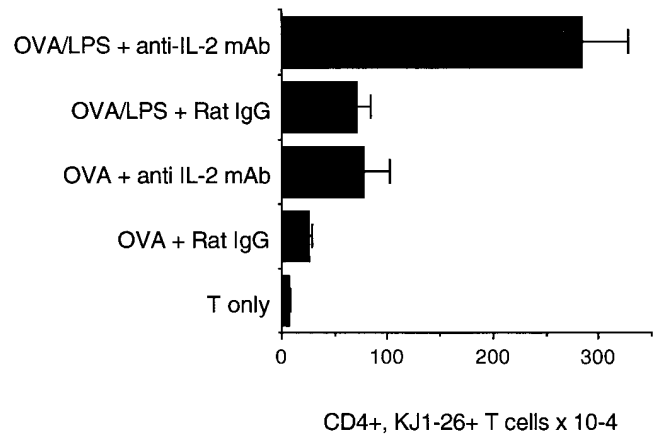


Figure 8. Expansion of Ag-specific T cells after injection of Ag with LPS is augmented in mice treated with anti-IL-2 mAb. Mice adoptively transferred with wild-type DO11.10 T cells were treated with anti-IL-2 mAb S4B6 or rat IgG and injected subcutaneously with 2 mg OVA and 150 μ g LPS. The number of CD4⁺, KJ1-26⁺ cells present in the draining LNs was determined on day 6. Each bar represents the mean \pm SE with five mice per group.

(25), and that LPS rapidly induces both molecules (9, 25). Therefore the suboptimal IL-2 production and clonal expansion that was observed in mice injected with OVA alone could be caused by Ag-presentation by B7-2^{low} resting dendritic cells, whereas the improved responses observed in the presence of LPS could be caused by B7-1⁺, B7-2^{high}-activated dendritic cells.

The small amount of IL-2 production and clonal expansion observed in the absence of CD28-mediated costimulation may be explained by other costimulatory molecules. Indeed, we (4) and others (26) have shown that inflammatory cytokines, such as TNF- α and IL-1, mimic the effects of LPS on the clonal expansion of T cells stimulated by Ag. However, the failure of LPS to enhance the clonal expansion of Ag-stimulated, CD28-deficient T cells indicates that TNF- α or IL-1 does not enhance T cell accumulation in a CD28-independent fashion as described in a superantigen model (27). The stimulatory effects of TNF- α and IL-1 on T cells may operate indirectly through induction of CD40, which has been shown to regulate B7 expression on APC (28).

In most cases, IL-2 production and clonal expansion were correlated, as would be expected if the former caused the latter. However, our finding that the initial clonal expansion of IL-2-deficient T cells was at least as large if not larger, than that exhibited by IL-2-sufficient T cells, demonstrates that IL-2 production is not necessary for maximal clonal expansion. This interpretation is supported by the observation of Kneitz et al. (19) that clonal expansion of superantigen-specific T cells is normal in IL-2-deficient mice. Thus, other growth factors may be more important for driving in vivo clonal expansion, although as discussed below IL-2 may participate. IL-15 is a candidate for such a growth factor because it partially restores proliferation of Ag-activated, IL-2R-deficient TCR transgenic T cells in vitro (23). However, residual in vitro proliferation of IL-2-deficient T cells in response to TCR stimulation does not

Table 2. Cytokine Profile of Naive and Primed Wild-type and IL-2-deficient DO11.10 T Cells

Cytokine level per DO11.10 T cell	Uninjected		OVA/LPS injected	
	WT SCID DO11.10 T cells	IL-2 KO CD45RB ^{high} DO11.10 T cells	WT SCID DO11.10 T cells	IL-2 KO CD45RB ^{high} DO11.10 T cells
IL-2 fg/ml ± SD	352.0	Undetectable	61.6 ± 8.3	Undetectable
IL-3 OD _u ± SD	374.0	432.0	59.4 ± 3.1	226.5 ± 37.5
IL-5 OD _u ± SD	Undetectable	Undetectable	5.9 ± 0.1	7.5 ± 1.1
IFN-γ fg/ml ± SD	Undetectable	Undetectable	300.0 ± 5.7	18.6 ± 1.6

In vitro cytokine production profiles of IL-2-sufficient and IL-2-deficient DO11.10 T cells after in vivo immunization with OVA and LPS. Normal BALB/c mice were transferred with IL-2-sufficient DO11.10 SCID cells or sorted IL-2-deficient CD45RB^{high} DO11.10 cells and were either injected with 2 mg OVA and 150 μg LPS or left uninjected. Mice were killed on 8 d, and LN cells were cultured with or without OVA peptide. Indicated cytokines were measured in 48-h culture supernatants as described in Materials and Methods. Cytokine concentrations were adjusted for the input number of CD4⁺, KJ1-26⁺ T cells present in each well. No detectable cytokine was seen in the absence of OVA peptide stimulation. The results of this experiment are representative of two other similar experiments where unsorted wild-type and IL-2-deficient DO11.10 T cells were used. WT, wild type.

depend on signals delivered through the common γ chain (γ_c) of the IL-2, IL-4, IL-7, IL-9, and IL-15 receptors (18), suggesting that signal(s) other than IL-15 may be involved. Because proliferation of IL-2-deficient T cells has been shown to be highly dependent on B7-derived costimulation (18), and γ_c-deficient T cells benefit from CD28 signaling (29), it is reasonable to suggest that the IL-2-independent growth signal is another cytokine whose production is CD28-dependent. Alternatively, CD28 signaling may drive proliferation directly or upregulate other surface molecules that deliver growth signals. Our finding that Ag-stimulated, IL-2-deficient T cells did not become IFN-γ-producing cells in vivo, together with a similar in vitro result with IL-2R-deficient T cells (23), indicate that IL-2 plays an important role in T cell differentiation.

The normal initial in vivo clonal expansion of Ag-activated, IL-2-deficient, DO11.10 T cells was surprising because of the poor in vitro proliferative capacity of these cells. It is difficult to believe that IL-2 is an important T cell growth factor in vitro, but not in vivo. It is possible that IL-2 is an important T cell growth factor in vivo at limiting antigen doses. However, at the high antigen dose used here, the growth factor function of IL-2 may be difficult to reveal because IL-2 also sensitizes activated T cells for cell death, perhaps as a consequence of the FasL-Fas interaction (19, 23). This model suggests that in the presence of IL-2, naive T cells would proliferate maximally because of the synergy between autocrine IL-2 and an IL-2-, γ_cR-independent growth signal, but would also become primed for programmed cell death. In the absence of IL-2, T cells would proliferate suboptimally because only the IL-2-independent growth signal would be available, but the cells

would survive better because of the absence of IL-2-driven cell death. The combination of reduced proliferation and absence of IL-2-driven cell death could explain why IL-2-deficient T cells accumulated to the same level as wild-type cells on day 3. After day 3, proliferation would cease and only T cells that were activated in the presence of IL-2 would be sensitive to death signals and die, while T cells that proliferated in the absence of IL-2 would persist. This would explain why on day 6 the number of IL-2-deficient DO11.10 T cells present in LNs was considerably higher than that of wild-type DO11.10 T cells. It is possible that the IL-2-dependent death signal is not delivered efficiently in vitro (few T cells undergo cell death after in vitro activation, data not shown). Thus, IL-2-deficient T cells would proliferate poorly in vitro because only the activity of the IL-2-independent growth factor would be present.

The paradoxical capacity of IL-2 to initially drive the proliferation of naive T cells, and then the death of activated T cells, was previously suggested by Lenardo et al. (30, 31) to provide a negative feedback mechanism that prevents excessive accumulation of activated T cells. It is possible that the lethal lymphoproliferative disease that occurs in IL-2-deficient mice that have a normal T cell repertoire is the result of non-IL-2 growth signal-driven T cell expansion unchecked by IL-2-mediated death. It is also interesting to note that this disease does not occur in germ-free mice (32). Intestinal microbial flora could provide not only a source of potentially cross-reactive Ags, but also a source of adjuvants, which based on our results with LPS, would heighten the production of B7-dependent, non-IL-2 growth signals. In the absence of IL-2, autoreactive T cells would then accumulate out of control due to lack of constraint provided by IL-2-mediated cell death.

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