

# Prevention of Peripheral Tolerance by a Dendritic Cell Growth Factor: Flt3 Ligand as an Adjuvant

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## Summary

Injections of soluble proteins are poorly immunogenic, and often elicit antigen-specific tolerance. The mechanism of this phenomenon has been an enduring puzzle, but it has been speculated that tolerance induction may be due to antigen presentation by poorly stimulatory, resting B cells, which lack specific immunoglobulin receptors for the protein. In contrast, adjuvants, or infectious agents, which cause the release of proinflammatory cytokines such as tumor necrosis factor  $\alpha$  and interleukin  $1\beta$  *in vivo* are believed to recruit and activate professional antigen-presenting cells to the site(s) of infection, thereby eliciting immunity. Here we show that administration of Flt3 ligand (FL), a cytokine capable of inducing large numbers of dendritic cells (DCs) *in vivo*, (a) dramatically enhances the sensitivity of antigen-specific B and T cell responses to systemic injection of a soluble protein, through a CD40–CD40 ligand–dependent mechanism; (b) influences the class of antibody produced; and (c) enables productive immune responses to otherwise tolerogenic protocols. These data support the hypothesis that the delicate balance between immunity and tolerance *in vivo* is pivotally controlled by DCs, and underscore the potential of FL as a vaccine adjuvant for immunotherapy in infectious disease and other clinical settings.

Key words: Flt3 ligand • dendritic cells • adjuvant • tolerance • immunity

A productive immune response against an invading pathogen occurs through the clonal expansion of functionally competent B and T lymphocytes that specifically recognize the pathogen through surface receptors for antigen (1). It is well established that soluble proteins do not induce productive immunity unless they enter the body with adjuvants or infectious agents (2–6). Expansion of antigen-specific T cells after exposure to soluble antigen is only transient and is followed by their subsequent deletion and/or functional unresponsiveness, a state that has been termed “clonal anergy” (7–10). It has been proposed that soluble protein antigens are presented to T cells by resting B cells, which lack critical costimulatory molecules on their surface and thus often induce immunological tolerance (11–14). Adjuvants or infectious organisms are thought to act by recruiting professional APCs to the site(s) of infection, and by directly stimulating these APCs to express costimulatory molecules (9, 15–17). The expression of costimulatory molecules, such as CD80 and CD86, on these cells bestows them with immunostimulatory capacities, and they are thus able to present antigen to T cells in an immunogenic fashion.

One cell type that is thought to play a crucial role in the initiation of immunity is the dendritic cell (DC)<sup>1</sup> (18, 19). By *in situ* staining, DCs located in the T cell zones of spleens and lymph nodes have been shown to express high levels of CD86 (20), compared with B cells and other APCs. Indeed, DCs by virtue of their excellent antigen capture, processing, and presentation capacities, and their mobility throughout the body, have been hailed as “nature’s adjuvants” (18). Research into DCs and the roles they play in the regulation of immune responses has been somewhat hampered by their rarity in tissues. One recent solution to this problem has been the identification of DC growth factors such as Flt3 ligand (FL), which has been shown to induce a profound expansion of mature DC subsets in various tissues in mice (21, 22). Such DCs constitutively express MHC class II antigens, CD86, and CD40, and are as efficient as DCs from untreated mice in priming antigen-specific T cells *in vitro* and *in vivo*. Here we inves-

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<sup>1</sup>Abbreviations used in this paper: CD40L, CD40 ligand; DC, dendritic cell; FL, Flt3 ligand; MSA, mouse serum albumin.

tigate the effect of FL on B and T cell responses to systemic injection of soluble proteins. We show that FL administration dramatically enhances antigen-specific antibody responses as well as T cell proliferation, through a mechanism involving CD40-CD40 ligand (CD40L) interaction. Furthermore, this treatment appears to prevent the establishment of peripheral T cell tolerance, which is induced by systemic injection of soluble antigens.

## Materials and Methods

**Mice.** DO11.10 TCR mice (23) were bred in a pathogen-free facility according to the National Institutes of Health guidelines. BALB/c and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and kept in a specific pathogen-free facility. For adoptive transfers, age- and sex-matched BALB/c recipients were given  $2.5 \times 10^6$  DO11.10 TCR transgenic T cells intravenously as described previously (8).

**Injections.** Mice (2–10/group) were injected once daily (subcutaneously at the nape of the neck) with 1  $\mu$ g mouse serum albumin (MSA) plus 10  $\mu$ g of recombinant human FL (human Chinese hamster ovary cell-derived) for 9 consecutive d. Chicken OVA (Sigma Chemical Co., St. Louis, MO) was freshly prepared in PBS, filtered through a 0.45- $\mu$ m pore size filter, and deaggregated by centrifugation at 15,000 *g* for 10 min. Footpad injections were given in a volume of 25  $\mu$ l. In the tolerance experiments, intraperitoneal injections were given in a volume of 200  $\mu$ l. OVA was emulsified in CFA (Sigma Chemical Co.), as described previously (8). The M158 antibody against murine CD40L was prepared at Immunex (Seattle, WA), and found to be endotoxin-free. This was injected in a volume of 25  $\mu$ l in the footpad. A rat IgG antibody (M132) made at Immunex was used as an isotype control.

**Flow Cytometry.** Cell suspensions were prepared from the popliteal draining lymph nodes and incubated on ice with PE-labeled anti-CD4 (PharMingen, San Diego, CA) and FITC-labeled KJ1-26 mAb (24), as previously described (8). A FACScan® flow cytometer (Becton Dickinson, San Jose, CA) was used to collect and analyze 20,000 events that had the light scatter properties of lymphocytes.

**In Vitro Cultures.** Various times after priming with OVA in the hind footpads,  $5 \times 10^5$  popliteal lymph node cells were plated in triplicate in 96-well flat-bottomed plates (Costar, Cambridge, MA) in 200  $\mu$ l of DMEM complete supplemented with 5% FBS, together with different concentrations of OVA 323–339 peptide. Proliferative responses were assessed after 72 h of culture in a humidified atmosphere of 5% CO<sub>2</sub> in air. Cultures with pulsed with 0.5  $\mu$ Ci [<sup>3</sup>H]thymidine for 5 h and the cells were harvested onto glass fiber sheets for counting on a gas-phase  $\beta$  counter. For cytokine assays, aliquots of culture supernatants were removed after 72 h, pooled, and assayed for the presence of IFN- $\gamma$ , IL-2, IL-4, and IL-10 by ELISA.

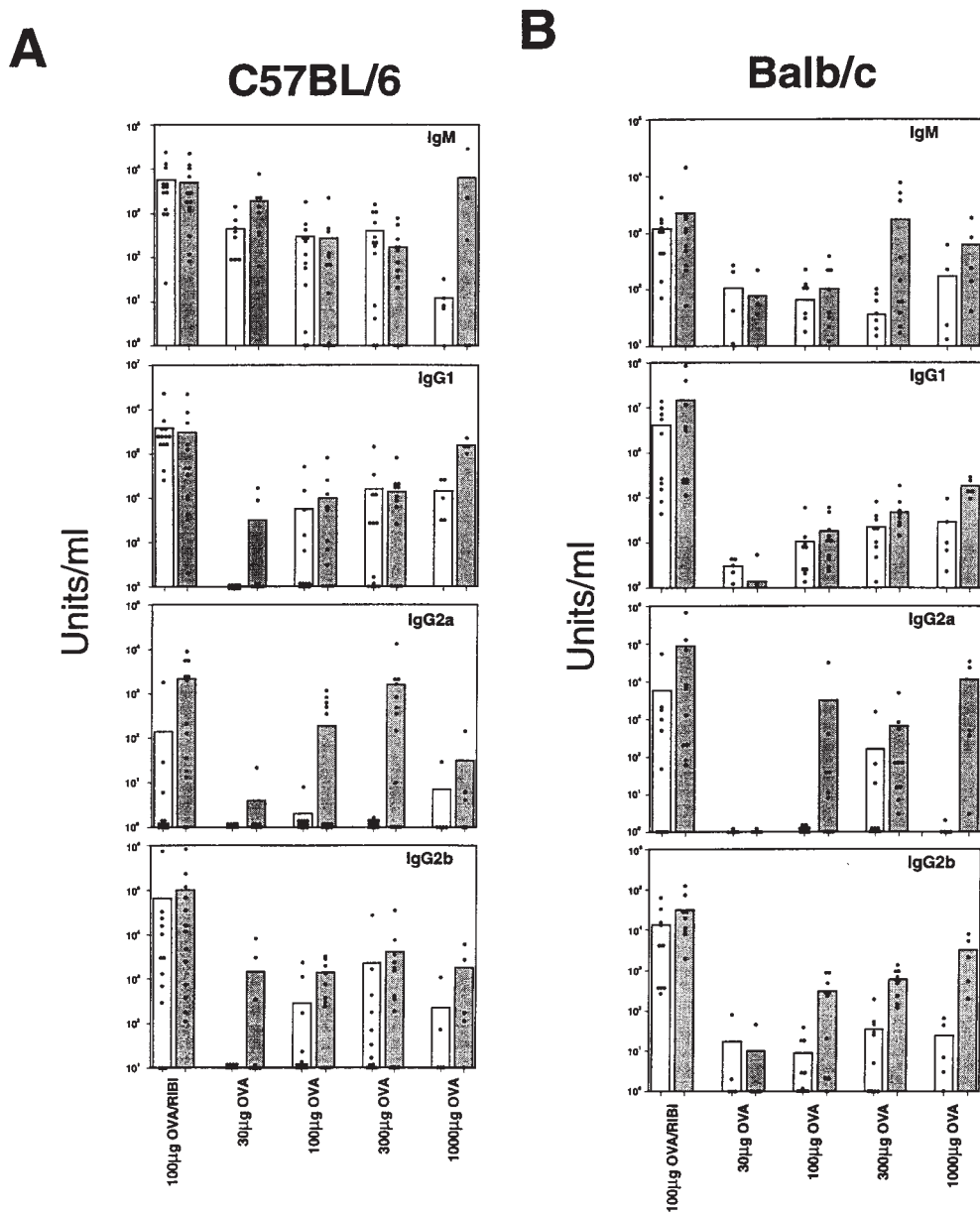
**Measurement of OVA-specific Serum Titers.** 96-well ELISA plates (Maxisorp; Nunc, Naperville, IL) were coated overnight with 1  $\mu$ g/well OVA in PBS at 4°C, blocked with PBS/5% FBS, and washed with PBS/0.1% Tween 20. Serum samples were diluted in PBS/5%FBS (starting at 1:100), and threefold dilutions were made. Plates were incubated for 2 h at room temperature, washed, and incubated with alkaline phosphatase-conjugated anti-IgG1 (1:2,000; PharMingen), anti-IgG2a, anti-IgG2b or anti-IgM (1:1,000; PharMingen) detecting antibodies for an additional 2 h at room temperature. Plates were washed again, and

enzyme activity was detected with *p*-nitrophenyl phosphate disodium (Sigma Chemical Co.). The amount of reaction product was assessed on an ELISA plate reader at an OD of 405 nm using the Deltasoft program (DeltaPoint, Monterey, CA). Multiple point analysis was performed on each set of isotype titrations using the BIOASSAY program (Immunex, Seattle, WA), selecting a maximum value for each isotype and determining for each sample the dilution giving half-maximal OD value, thus generating arbitrary unit per milliliter values as previously described (24a).

**Cytokine ELISAs.** IFN- $\gamma$ , IL-2, IL-10, and IL-4 were quantified by ELISAs adapted from PharMingen protocols. In brief, Nunc ELISA plates (Maxisorp; Nunc) were coated with 100  $\mu$ l of the following antibodies diluted in 0.1 M NaHCO<sub>3</sub> for 6 h: R46A2 (mIFN- $\gamma$  at 1  $\mu$ g/ml); 11B11 (mIL-4 at 1  $\mu$ g/ml); and purified anti-mIL-2 and anti-mIL-10 (PharMingen) at 2  $\mu$ g/ml. The plates were then washed in PBS/0.1% Tween 20 and blocked with PBS/5% FBS for 6 h. Serial dilutions (twofold) of the samples were made in PBS/5% FBS and added to the plate overnight at 4°C. Twofold, serial dilutions of the standards (PharMingen) were made at the following starting concentrations: mIFN- $\gamma$  (18 ng/ml); mIL-4 (500 pg/ml); mIL-10 (10 ng/ml); mIL2 (10-ng/ml). After overnight incubation, the plates were washed, and biotinylated anti-cytokine antibodies (PharMingen) were added at the following dilutions: mIFN- $\gamma$  at 0.5  $\mu$ g/ml; mIL-4 at 0.25  $\mu$ g/ml; mIL-10 at 10 ng/ml; mIL-2 at 10 ng/ml. 2 h later the plates were washed and incubated with streptavidin-horseradish peroxidase (Zymed, San Francisco, CA) diluted in PBS/Tween 20 at 1:2,000. 2 h later, the plates were washed and the bound peroxidase was detected with TMB Microwell substrate (Kirkegaard & Perry Labs., Gaithersburg, MD). The amount of reaction product was assessed on an ELISA plate reader at an OD of 450 nm. The detection limits were: 100 pg/ml for IFN- $\gamma$ , 100 pg/ml for IL-2, and 20 pg/ml for IL-10.

## Results and Discussion

**FL Administration Dramatically Enhances Antibody Responses against Soluble Proteins.** We examined whether FL-treated mice had enhanced potential to mount antigen-specific immune responses against soluble antigens. C57BL/6 or BALB/C mice were treated with either FL or MSA (control) for 9 d. This FL treatment regimen results in up to a 30-fold expansion of DCs in spleen and various other tissues (21, 22). These animals were then injected subcutaneously with various doses of soluble OVA or OVA in the adjuvant RIBI (OVA/RIBI) on the final day of FL treatment. 3 wk later, the mice were boosted with a second injection of OVA or OVA/RIBI and the serum anti-OVA antibody titers were measured 7 d later. FL treatment dramatically enhanced IgG2a anti-OVA antibody titers in both C57BL/6 and BALB/C mice, compared with MSA-treated controls (Fig. 1, A and B). The titers of IgG2a OVA-specific antibody in FL-treated mice were, in some cases, increased up to 10,000-fold, and were as high as those in mice immunized with OVA/RIBI. In the BALB/C strain, in two experiments, a minor subset of the MSA-treated mice showed a significant IgG2a response when injected with 300  $\mu$ g of soluble OVA (Fig. 1 B). A lower (but detectable) level can also be seen in a single C57BL/6 mouse, in response to 100  $\mu$ g of OVA. In both strains, the



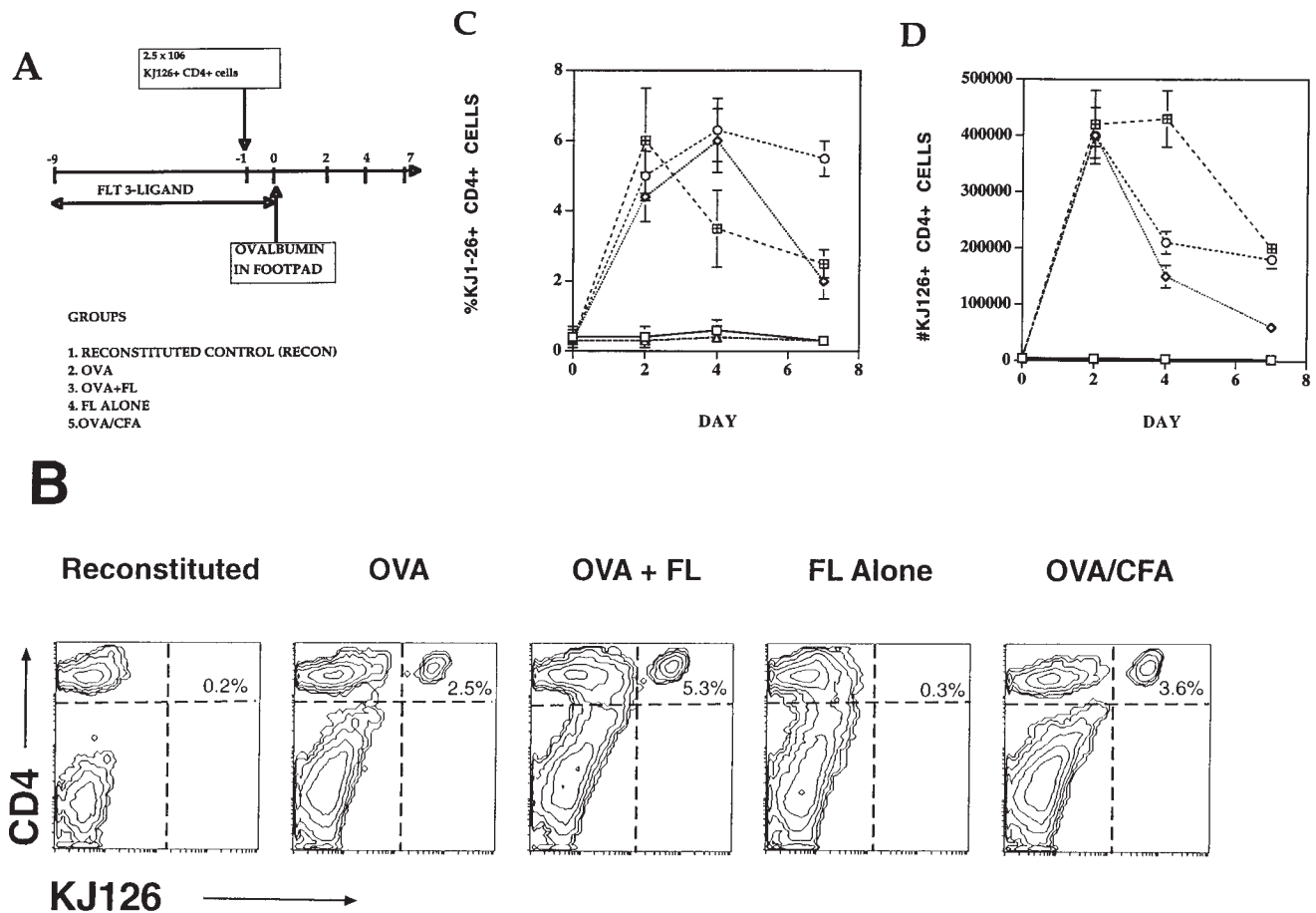
**Figure 1.** Antigen-specific, secondary serum antibody titers in FL-treated (gray bars) versus MSA-treated (control; white bars) C57BL/6 (A) and BALB/c (B) mice after subcutaneous OVA challenge. Data are derived from five independent experiments. Each dot represents a single mouse and histograms represent the arithmetic means.

minor subsets of responders gave titers above the threshold of detectability in this assay ( $<5$  U/ml). The reason(s) for this aberrant response is at present unclear, but may simply reflect variability between mice.

IgG1 anti-OVA antibody titers in both strains of FL-treated mice were elevated 3–10-fold over control mice at the 1,000- $\mu$ g dose of antigen ( $P < 0.01$ ). However, little to no enhancement of IgG1 was observed at lower antigen doses, with the exception of a subset of responders at the lowest antigen dose in FL-treated C57BL/6 mice. The IgM titers also showed increases at the higher doses. There were also marked increases in the IgG2b anti-OVA titers in FL-treated BALB/C mice, but more modest increases in the C57BL/6 strain. The IgE and IgG3 titers were unaffected by FL treatment (data not shown). Titers of total Ig

(IgM, IgG1, IgG2a, IgG2b, IgG3, and IgE) were unaffected by FL treatment, suggesting that the effect of FL occurred in an antigen-specific way (data not shown), with a preferential skewing towards an IgG2a response.

*FL Injection Enhances the Clonal Expansion and Proliferative Capacity of Antigen-specific T Cells In Vivo and In Vitro.* To investigate the effect of FL treatment on antigen-specific T cell responses, we used TCR transgenic mice that contain rearranged TCR- $\alpha$  and TCR- $\beta$  genes encoding a TCR specific for OVA 323–339 bound to I-A<sup>d</sup> class II MHC molecules (DO11.10 mice; reference 23). TCR transgenic T cells were adoptively transferred into syngeneic BALB/C recipients, such that they constituted a small but detectable proportion of all T cells (8, 9). In this system, the fate of the OVA-specific, transgenic T cells could



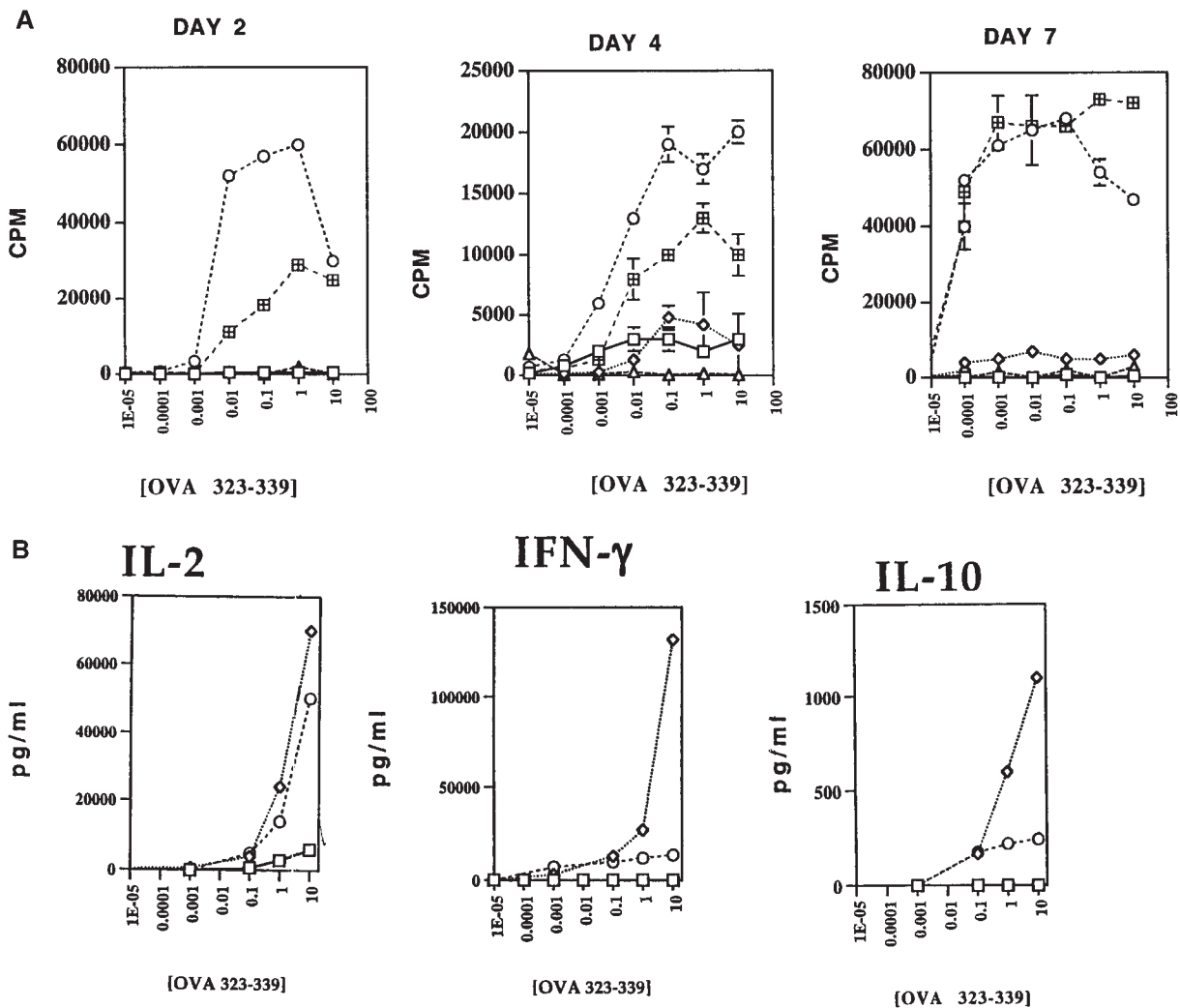
**Figure 2.** Effect of FL on antigen-specific T cell responses. (A) Experimental design. Mice were treated for 10 d (from day  $-9$  to  $0$ ) with FL or MSA, and  $10$  mmol ( $423 \mu\text{g}$ ) of OVA was injected in the footpads on day  $0$ . The draining popliteal lymph nodes were removed for phenotypic and functional analysis at days  $2$ ,  $4$  and  $7$ . (B) Flow cytometry profiles (CD4 versus KJ126) from day  $5$  of the response from a representative experiment. (C and D) The kinetics of expansion of antigen-specific T cells in vivo. At day  $7$  there is a significant enhancement in frequency (C) and absolute numbers (D) of antigen-specific T cells in FL-treated mice ( $P < 0.01$ ).  $\square$ , recon;  $\diamond$ , OVA;  $\circ$ , OVA + FL;  $\triangle$ , FL; *quartered square*, OVA/CFA. Data are representative of  $10$  experiments.

be followed with the KJ126 clonotypic antibody (24). The reconstituted mice were injected with  $10$  mmol OVA in the footpad, in either the presence or absence of FL treatment (Fig. 2 A). The CD4<sup>+</sup>, OVA-specific T cell response in the draining lymph node was monitored by flow cytometry. Injection of OVA elicited a significant clonal expansion of the KJ126<sup>+</sup>CD4<sup>+</sup> cells in the draining lymph nodes, consistent with previous reports (9). FL treatment significantly enhanced the percentage of KJ126<sup>+</sup>CD4<sup>+</sup> cells in vivo,  $7$  d after challenge ( $2.1\%$  soluble OVA versus  $5.9\%$  soluble OVA + FL; Fig. 2, B and C). There was a corresponding increase in the absolute numbers of KJ126<sup>+</sup>CD4<sup>+</sup> cells at day  $7$  (Fig. 2 D). Thus, FL treatment enhanced the clonal expansion of antigen-specific T cells induced by administration of a soluble protein antigen. This two- to threefold increase in the frequency and the absolute numbers of such cells is similar to the enhancement observed with a natural adjuvant such as LPS in this adoptive transfer system (9, 25). Interestingly, this increase is apparent only at day  $7$ , when the response is on the decline. Therefore, it is conceivable that FL is somehow retarding the elimina-

tion of cells rather than, or in addition to, enhancing their expansion.

FL treatment alone, in the absence of OVA injection, failed to elicit any response above the basal levels. The absolute number and activation state (assessed by CD69, CD44, CD45Rb, and CD62L expression), of the non-antigen-specific (KJ126<sup>-</sup>CD4<sup>+</sup>) T cells were unaffected by FL treatment (data not shown), suggesting that FL treatment enhances T cell responses in an antigen-specific manner.

Previous work in this system has shown that productive T cell immunity is elicited only when the antigen is injected with an adjuvant, and that injections of soluble antigens only result in a transient and abortive clonal expansion, in which the antigen-specific T cells cannot be efficiently restimulated in vitro with peptide (8, 9, 25). Therefore, we examined the in vitro proliferative capacity of the OVA-specific T cells from the mice injected with OVA (with or without FL) by culturing single-cell suspensions of the draining lymph nodes from the various cohorts of mice, in the presence of varying concentrations of OVA 323–339 peptide. As shown in Fig. 3 A, mice that received



**Figure 3.** (A) In vitro restimulation of antigen-specific T cells expanded in vivo (by injecting 10 mmol OVA in the footpad, as described in the legend to Fig. 2) with varying concentrations of OVA 323-339 peptide. □, recon; ◇, OVA; ○, OVA + FL; △, FL alone; ◻, OVA/CFA. (B) Cytokine production by antigen-specific T cells restimulated in vitro with varying concentrations of OVA 323-339 peptide. In vitro cultures were set up 6 d after priming in vivo. Culture supernatants were harvested 72 h later and assayed for cytokines by ELISA. The detection limits were 100 pg/ml for IFN- $\gamma$  and IL-2 and 20 pg/ml for IL-10. □, OVA; ◇, OVA + FL; ○, OVA/CFA. Data are representative of 10 (A) and 3 (B) independent experiments.

an injection of OVA without FL treatment had greatly diminished responses compared with those that received FL treatment, or those challenged with OVA/CFA, at each of the time points examined. As observed previously, injection of soluble OVA renders the mice unresponsive to an in vitro challenge with OVA 323-339 (8). However, FL treatment prevented this unresponsiveness (Fig. 3 A). Furthermore, FL treatment alone (in the absence of OVA) did not increase the proliferative capacity of T cells above the background controls. This suggests that the observed adjuvant effects of FL are indeed antigen-specific, and not caused by some generic effect of FL on all T cells. It should be noted that the concentration of OVA 323-339 peptide required to elicit a half maximal response diminished with time. Thus, at day 2, half maximal response was achieved with 0.01  $\mu$ M of peptide, whereas at day 6 only 0.0001  $\mu$ M peptide was required (Fig. 3 A). This may reflect a

state of transient refractoriness in the T cells after antigen-mediated stimulation in vivo.

Consistent with their increased proliferative capacity, OVA-specific T cells from mice injected with OVA plus FL made significantly higher levels of IL-2, IFN- $\gamma$ , and IL-10, when re-stimulated in vitro (Fig. 3 B). IL-4 was not detectable. The enhanced IFN- $\gamma$  production is consistent with the dramatic increase in OVA-specific IgG2a titers observed (Fig. 1), and is likely to be mediated by IL-12 produced by the DCs in vivo (22, 26, 27).

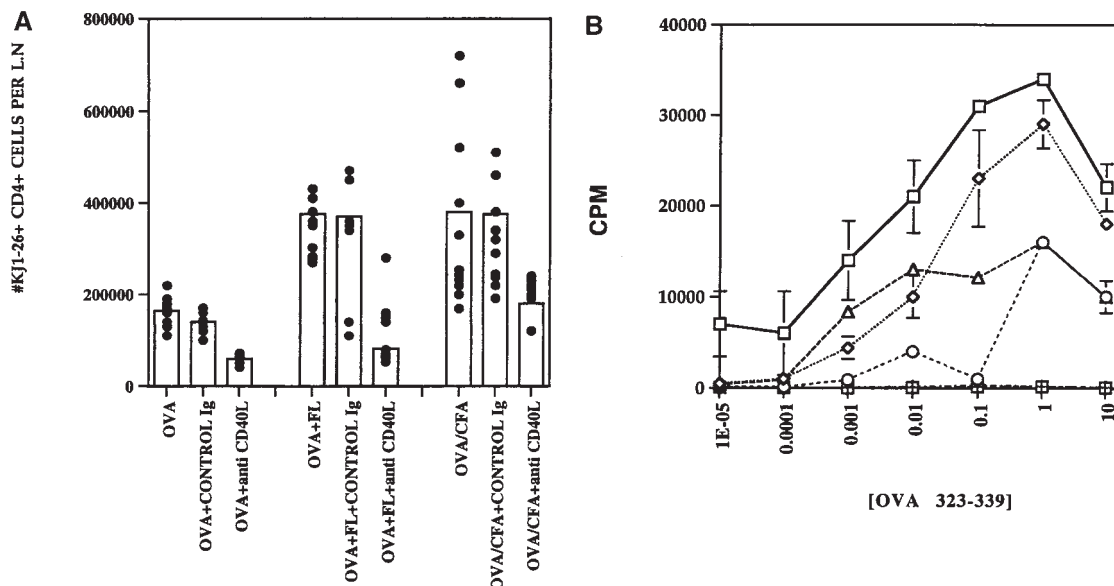
*A Role for CD40-CD40L Interaction in the Immune-enhancement Effects of FL.* There is strong evidence that CD40-CD40L interaction is crucial for humoral and cellular immune responses (28, 29). We examined the potential role CD40-CD40L interaction could play in mediating the enhanced immune responses, since DCs from FL-treated mice express significant levels of CD40 (21, 22). FL- or

MSA-treated mice were injected in the footpad with OVA (Fig. 2 A), and on days  $-1$ ,  $0$ ,  $+1$ , and  $+2$   $200 \mu\text{g}$  of the M158 antibody (antagonistic against murine CD40L) was injected intraperitoneally. Antigen-specific T cell responses were measured on day 5. As shown in Fig. 4 A, treatment with anti-CD40L mAb, but not the control antibody, significantly blunted the clonal expansion of  $\text{KJ126}^+\text{CD4}^+$  cells in vivo. The dramatic effect that FL treatment has on the in vitro proliferation of OVA-specific T cells is also greatly reduced as a result of blocking the CD40-CD40L pathway in vivo (Fig. 4 B). This is consistent with reports ascribing a pivotal role to CD40-CD40L interaction in the onset of T cell-dependent immune responses (30-33).

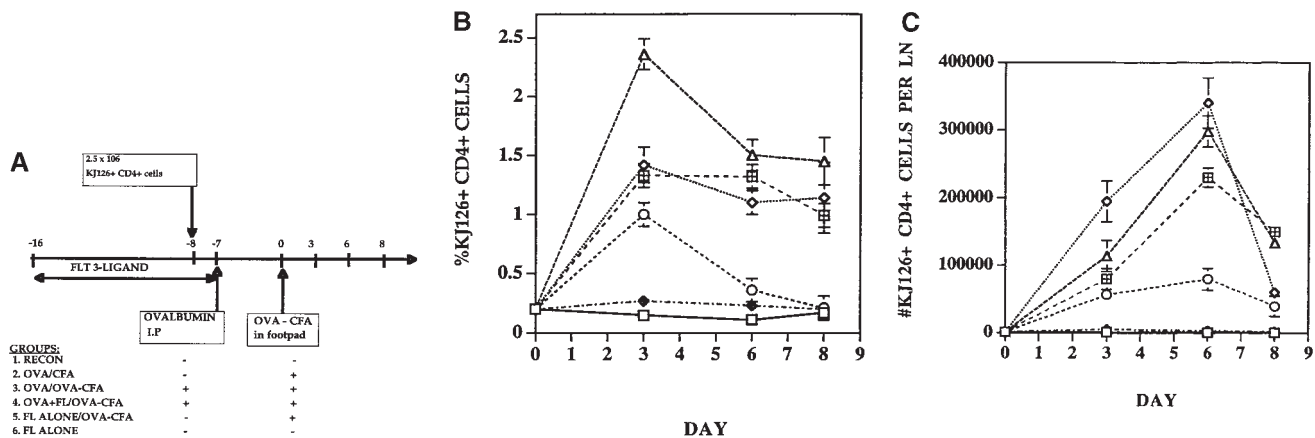
*FL Is Able to Prevent the Establishment of Peripheral Tolerance Caused by Systemic Injection of a Soluble Antigen.* Injection of soluble antigens into mice is known to induce antigen-specific T and B cell tolerance rather than immunity (2-6, 9). It has been proposed that this outcome may be due to antigen presentation to T cells by poorly stimulatory, resting B cells (11, 12, 14). Since the profound expansion in DCs caused by FL treatment dramatically enhances antigen-specific T and B cell responsiveness to soluble antigens, we investigated whether FL treatment could prevent the tolerance induction caused by systemic injection of soluble antigen. BALB/C mice, which had been reconstituted previously with transgenic T cells, were injected intraperitoneally with 1 mg of OVA, in either the presence or absence of FL treatment (Fig. 5 A). 7 d later, mice were challenged with OVA in CFA (OVA/CFA) in the footpads. The OVA-specific T cell response was monitored by flow cytometry as described above. OVA/CFA elicits an anti-

gen-specific T cell response, and, as shown previously (8), injection of OVA before challenge with OVA/CFA induces a state of impaired responsiveness, evidenced by a reduction in the frequency and the absolute numbers of  $\text{KJ126}^+\text{CD4}^+$  cells, compared with the OVA/CFA controls (Fig. 5, B and C). In contrast, mice that received OVA in the presence of FL treatment responded even better than the mice that had not been pretreated with OVA before challenge immunization with OVA/CFA. Therefore FL treatment appears to prevent the nonresponsiveness caused by soluble antigen.

Thus the in vivo expansion in DCs caused by FL treatment is able to enhance immune responses dramatically against a soluble antigen via a mechanism involving CD40-CD40L interaction, and bypass the induction of peripheral tolerance caused by systemically administered soluble antigen. These results are consistent with the recently described ability of FL to augment both antitumor immunity (34) and rejection of allogeneic transplants (35). They also support the recent observations of Gong et al. (36) that hybridoma DCs can abrogate T cell tolerance to tumor antigens in transgenic mice. However, our results differ from a recent report that describes the effect of FL in enhancing oral tolerance to fed soluble antigens at certain low doses of antigen (37). The contrasting effects of FL treatment on T cell tolerance to orally versus systemically administered antigens is reminiscent of the effects that LPS has on tolerance induced by these different routes of antigen administration. Although LPS is known to abrogate peripheral tolerance (8, 17), several reports describe its ability to enhance oral tolerance (e.g., 38). The reason(s) for the differences between



**Figure 4.** Effect of in vivo CD40-CD40L blockade on the expansion of antigen-specific T cells in vivo and in vitro. (A) The blockade of CD40-CD40L interaction in vivo with the M158 antibody ( $200 \mu\text{g}$  administered intraperitoneally on days  $-1$ ,  $0$ ,  $+1$ , and  $+2$ ) significantly blunts the clonal expansion of antigen-specific T cells in FL-treated mice and OVA/CFA-immunized mice. Each dot represents a single mouse and histograms represent the arithmetic means. (B) Upon restimulation in vitro with varying concentrations of OVA 323-339 peptide, there is a profound impairment of proliferative capacity in mice that received the M158 antibody. □, OVA + FL; ◇, OVA + FL + control IgG; ○, OVA + FL + anti-CD40L; △, OVA/CFA + control IgG; quartered square, OVA; ◆, OVA + control IgG. Data pooled from two independent experiments in which three to five mice per group were used.



**Figure 5.** Effect of FL treatment on the induction of peripheral tolerance caused by systemic injections of soluble antigens. (A) Experimental design. Mice were tolerized with 1 mg of soluble, deaggregated OVA, and challenged with OVA/CFA 7 d later. B and C show the kinetics of expansion frequency and absolute number, respectively, of antigen-specific T cells in vivo. □, recon; ◇, OVA/CFA; ○, OVA-OVA/CFA; △, OVA + FL-OVA/CFA; quartered square, FL-OVA/CFA; ◆, FL alone. Data are pooled from six independent experiments, with a total of 6–35 lymph nodes per time point.

our observations and those of Viney et al. (37) are unclear at present, but may well reside in the functional differences in DC subsets, or in differences in the microenvironments between the gut and peripheral lymphoid organs.

The exact mechanism by which FL treatment enhances immune responses is unclear. It is useful to consider this question in the context of the recent observation that the establishment of T cell anergy by soluble antigens in vivo may be dependent on the interaction between B7 molecules on APCs and CTLA-4 on T cells (39). This mechanism of T cell tolerance is likely to occur when the APC expresses very low levels of B7 molecules, resulting in a

preferential interaction with the high affinity CTLA-4 receptor rather than with CD28. In FL-treated mice, there is an enormous expansion of CD86<sup>+</sup> DCs in the T cell zones and marginal zones of spleens (22). It is possible that this increased surface area and density of CD86<sup>+</sup>CD40<sup>+</sup> class II<sup>+</sup> APCs in the T cell zones alters the balance between tolerance and immunity and results in soluble antigen being presented to T cells in an immunogenic way.

In summary, these data provide evidence for DCs playing a pivotal role in maintaining the equilibrium between immunity and self-tolerance in vivo, and highlight the potential for FL as a vaccine adjuvant.

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