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# INTERLEUKIN 1 ENHANCES T-DEPENDENT IMMUNE RESPONSES BY AMPLIFYING THE FUNCTION OF DENDRITIC CELLS

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Evidence that soluble factors are important in T cell mitogenesis began with experiments in which leukocyte-conditioned media enhanced growth in the presence of lectin (1, 2). Two factors were identified in these media (3): IL-1, a macrophage product originally termed lymphocyte activating factor and IL-2, a T cell product originally termed T cell growth factor. A longstanding hypothesis has been that T cells proliferate when exposed to antigen (or polyclonal stimuli like lectins) plus IL-1, with both signals originating from accessory or antigen-presenting cells (4). It has been further proposed that IL-1 acts directly on T cells either to induce IL-2 release (5, 6) or responsiveness (7). Much of the supporting evidence for these ideas has derived from selected T cell lines and leukemias (7, 11), subpopulations of thymocytes (5, 12), or from primary T cell populations that may not have been rigorously depleted of accessory cells (5, 6).

Recent advances have made it possible to analyze the function of IL-1 on primary lymphocytes. A purified recombinant IL-1 (rIL-1) has been produced that has the same biologic activity as macrophage-derived IL-1 (13). The cells involved in T-dependent responses also are better characterized. These include: dendritic cells (DC),<sup>1</sup> which are the principal accessory cells for the growth of resting helper lymphocytes (14–17); primed antigen-specific T lymphoblasts, which actively release and respond to IL-2 (18); and memory T cells, which are derived from the blasts but are no longer IL-2 responsive (19). We have examined the effects of rIL-1 on DC and on T lymphocytes in different states of activation. We find that IL-1 has little direct effect on peripheral T cells and does not synergize with antigen to initiate T-dependent responses. Instead, the amplifying role of IL-1 is due to an effect on the accessory function of DC, which increases substantially after exposure to IL-1 but not to other cytokines.

## Materials and Methods

### *Mice*

6–10-wk-old mice of both sexes were obtained from the Trudeau Institute, Saranac Lake, NY [B6.H-2k; (C × D2)F<sub>1</sub>; C3H/HeJ; (C57BL/6 × DBA/2)] and the Rockefeller University (BALB/c/AnN; NCS).

<sup>1</sup> *Abbreviations used in this paper:* DC, dendritic cell; EA, antibody-coated erythrocytes; TNF, tumor necrosis factor.

### Cytokines

**IL-1.** Murine rIL-1 $\alpha$  was generously provided by Dr. P. LoMedico, Hoffman LaRoche, Nutley, NJ. The IL-1 was purified from *E. coli* expressing the pIL-1 (1–156) plasmid (13) and had a specific activity of  $6 \times 10^6$  U/mg in the standard mitogenesis assay, in which thymus cell suspensions are stimulated with PHA and IL-1 (5). IL-1 was stored at  $2 \times 10^6$  or  $0.4 \times 10^6$  U/ml, and was used at final concentrations of 0.005–150 U/ml. A control preparation at 0.4 mg/ml was obtained from *E. coli* lacking the IL-1 cDNA insert. The D10 G4.1 T cell clone (7) (provided by Dr. C. Janeway, Yale Univ., New Haven, CT) was used as a more sensitive indicator for IL-1. Whereas half-maximal thymocyte costimulation was achieved with 1–5 U/ml rIL-1, half-maximal D10 mitogenesis occurred at 0.01–0.1 U/ml (0.1–1.0 pM). The D10 cells were propagated in culture by stimulating  $10^6$  cells with  $5 \times 10^6$  irradiated (6,000 rad  $^{137}\text{Cs}$ ) B6.H-2k spleen cells and 100  $\mu\text{g/ml}$  conalbumin (Sigma Chemical Co., St. Louis, MO) in 10 ml medium in 100-mm culture dishes. Four or more days later, the D10 cells were isolated on Histopaque (Sigma Chemical Co.; specific gravity of 1.119) columns (two or three Petri dishes of cells on 20 ml, 500 g for 20 min at room temperature). Viable cells at the interface were cultured overnight before use in the bioassay.  $2\text{--}3 \times 10^4$  D10 were plated in flat-bottomed microtest wells (Costar, Cambridge, MA) with serial dilutions of IL-1 or viable DC in a total volume of 0.1 ml for 3 d. Proliferation was assessed after culture in 0.5  $\mu\text{Ci}$  [ $^3\text{H}$ ]TdR (Schwartz Mann, Orangeburg, NY; 6.7 Ci/mM) for 16 h on day 2–3. D10 cells did not require the addition of Con A to respond to IL-1 and also did not respond to LPS.

**rIL-2.** Human rIL-2 at  $8.5 \times 10^5$  U/mg was provided by Biogen Corp., Cambridge, MA. IL-2 activity in subsaturating volumes of medium was measured using Con A-stimulated lymphoblasts as responders.  $5 \times 10^6$  lymph node cells in 5 ml medium were stimulated for 3 d with 1  $\mu\text{g/ml}$  Con A (Pharmacia Fine Chemicals, Uppsala, Sweden).  $2 \times 10^4$  washed lymphoblasts were then cultured with graded doses of IL-2 for 24 h, and proliferation was measured with a 4–6 h pulse of 1.75  $\mu\text{Ci}$  [ $^3\text{H}$ ]TdR. Human IL-2 gave half-maximal stimulation of mouse Con A blasts and the D10 line at 3–10 U/ml.

**rIFN- $\gamma$ .** Murine rIFN- $\gamma$  at  $6 \times 10^6$  U/mg was provided by Genentech, South San Francisco, CA. 1 U/ml for 48-h optimally activated mouse peritoneal macrophages to release  $\text{H}_2\text{O}_2$  (20) and to express Ia antigens (21, 22). To induce Ia, we cultured elicited (1 ml Brewers' thioglycollate; Difco, Detroit, MI) peritoneal macrophages ( $0.7\text{--}7 \times 10^4$  cells per 12-mm circular glass coverslips; Propper Mfg. Co., Long Island, NY) for 3 d, with or without IFN- $\gamma$  at 10 U/ml. Elicited but not resident peritoneal cells responded to IFN- $\gamma$  as described (22). Induction of Ia antigens was visualized by staining cells with hybridoma culture supernatants (American Type Culture Collection, Bethesda, MD): 10-2.16 anti-Ia<sup>k</sup> (TIB 93) followed by FITC-goat anti-mouse Ig, B21-2 anti-Ia<sup>d</sup> followed by FITC-mouse anti-rat Ig, or biotin, 14-4-4S anti-I-E<sup>d,k</sup> (HB 32) followed by FITC-avidin. Staining was monitored by immunofluorescence microscopy and judged to be specific through the use of an isotype-matched control mAb or with cells from the inappropriate haplotype (22).

**Recombinant cachectin/tumor necrosis factor (TNF).** Human recombinant cachectin was synthesized in a yeast expression system by Chiron, Emeryville, CA. Recombinant cachectin was purified and provided by Dr. B. Beutler, the Rockefeller University. The specific activity was  $10^8$  U/mg in the L929 cytolysis assay (23) and had 0.4  $\mu\text{g/mg}$  LPS activity in the limulus amebocyte assay.

**Conditioned media.** Several media were used as natural sources of cytokines, as established in the above bioassays and in an assay for IL-3 (24). The media were from the J774 macrophage cell line stimulated with LPS (IL-1 and TNF); WEHI-3 cell line (IL-3); keratinocytes from murine epidermis (25), which release activities similar if not identical to IL-1 and IL-3 (26, 27). rIL-2, recombinant cachectin, and WEHI-conditioned media did not induce the release of IL-1 (D10 bioassay) from spleen macrophages, which indicates that these preparations had  $<1$  ng/ml LPS.

**Culture medium.** RPMI-1640 (Gibco, Grand Island, NY) was supplemented with 10% heat-inactivated FCS (Hyclone, Logan, UT), 1 mM glutamine (KC Biologicals, Inc.,

Lexena, KS), 100  $\mu\text{g/ml}$  streptomycin and 100 U/ml penicillin (Gibco), 20  $\mu\text{g/ml}$  gentamycin (Gibco) and  $5 \times 10^{-5}$  M 2-ME.

#### *T Cells and T Cell Proliferation*

*Unprimed peripheral T cells.* Unprimed peripheral T cells were nylon wool–nonadherent, spleen, and lymph node suspensions that were treated with anti-Ia mAb (10-2.16 anti-I-A<sup>k</sup> or B21-2 anti-I-A<sup>d</sup>) and pooled rabbit complement (Pel-Freeze, Brown Deer, WI) to deplete residual B cells and DC. In some experiments (see Results), anti-Lyt-2.2 mAb (TIB 150, American Type Culture Collection) was used to deplete the cytolytic T cell subset, and J11d (TIB 183, American Type Culture Collection) to further deplete accessory cells. J11d kills purified DC and reduces the residual Con A responsiveness of anti-Ia and complement–treated, nylon-nonadherent cells. T cell proliferation was followed in the syngeneic and allogeneic MLR, and the polyclonal response to 1  $\mu\text{g/ml}$  Con A or 1.2 mM sodium periodate (14–16). In each, graded doses of accessory cells were added to  $3 \times 10^5$  responder T cells in 0.2 ml medium in flat-bottomed 96-well plates, or  $3 \times 10^6$  T cells in 2 ml medium with 24-well plates. 1  $\mu\text{Ci}$  [<sup>3</sup>H]TdR in 50  $\mu\text{l}$  medium was added for 16 h at the times indicated in the Results. Data are means of triplicates in which standard deviation ranged from 5–20% of the mean. All experiments were repeated at least once.

*Freshly sensitized T lymphoblasts.* Freshly sensitized T lymphoblasts were induced by allogeneic DC as described (18). The blasts doubled ( $t_{\text{gen}}$  of 14–16 h) in response to IL-2.

*Memory T cells.* Memory T cells were prepared by resting the blasts for three or more days in the absence of exogenous IL-2 or alloantigen (19). The blasts and memory populations were >70% allospecific, as assessed in rapid DC–T binding assays (28). Proliferative responses were measured with 1.75  $\mu\text{Ci}$  [<sup>3</sup>H]TdR applied for 6–8 h at the indicated times.

#### *Primary Antibody Responses*

Mixtures of accessory–cell depleted B and T lymphocytes were obtained by passing spleen suspensions over Sephadex G10 (17).  $4 \times 10^6$  lymphocytes were cultured in 16-mm-diam wells for 4 d with antigen ( $2 \times 10^6$  SRBC; or 10  $\mu\text{g/ml}$  TNP-KLH) and graded doses of DC (17). PFC were measured on both SRBC and TNP-SRBC to establish the antigen specificity of the PFC response.

#### *Accessory Cells*

Low-density spleen adherent cells were separated into DC- and macrophage-rich components by rosetting with antibody-coated erythrocytes (EA) (29). The EA<sup>+</sup>, DC-enriched populations were >90% DC. The EA<sup>+</sup> fraction had roughly equal numbers of macrophages and B cells and a small (3–10%) percentage of DC. DC were identified on the basis of irregular cell shape, and absence of Fc receptors, and macrophage/lymphocyte surface markers (29). The level of DC Ia was monitored on a FACS IV (Becton Dickinson Immunocytometry Systems, Mountain View, CA) after staining with FITC–B21-2 anti-Ia. To inactivate the accessory function of DC, we exposed the cells to a UV lamp (General Electric, 15 W; G15T8) for 2–4 min at a distance of 10 cm. Ia<sup>+</sup> and Ia<sup>−</sup> macrophages were obtained by culturing thioglycollate-elicited peritoneal cells for 3 d with and without rIFN- $\gamma$  as described above. The macrophages were cultured in graded doses on 12-mm coverslips, or the exudates were maintained in suspension in Teflon beakers (32) and harvested 3 d later. 1  $\mu\text{g/ml}$  indomethacin was added to all T cell responses in which macrophages were used as accessory cells.

#### *Dendritic–T Cell Clustering*

To follow the clustering of DC with T cells during the MLR, we labeled the DC with a nontoxic, stable, carbocyanine dye (30). We applied 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (diI) (Molecular Probes, Junction City, OR) at 20  $\mu\text{g/ml}$  to purified DC for 1 h at 37°C. This treatment did not alter the stimulatory function of DC, which clustered with helper T cells early in the MLR as described (18).

TABLE I  
*Effects of rIL-1 and rIL-2 on T Cell Proliferation and IL-2 Release: Comparison with DC*

Stimulus applied to T cells	Response (cpm $\times 10^{-3}$ ) of Lyt-2 <sup>-</sup> T cells			
	Unprimed	Primed blasts	3-d memory	7-d memory
<u>Proliferative response</u>				
None	0.2	1.1	0.3	0.3
10 <sup>4</sup> DC	26.1	60.8	282.1	210.1
10 <sup>3</sup> DC	2.0	39.5	179.8	133.3
10 <sup>2</sup> DC	0.5	14.8	52.9	44.7
rIL-2, 125 U/ml	0.6	42.7	8.8	3.7
rIL-1, 25 U/ml	0.4	1.0	0.7	0.4
rIL-2 + rIL-1	1.4	57.3	9.1	12.1
<u>IL-2 in medium</u>				
None	0.4	1.2	0.5	0.5
10 <sup>4</sup> DC	19.0	48.6	40.2	50.4
10 <sup>3</sup> DC	1.7	14.8	16.3	44.4
10 <sup>2</sup> DC	0.7	2.7	5.1	15.6
rIL-2, 125 U/ml	30.6	7.8	9.0	27.5
rIL-1, 25 U/ml	0.5	1.3	0.6	0.5
rIL-2 + rIL-1	29.0	ND	6.2	13.6

The numbers and culture intervals for the different T cells were:  $3 \times 10^5$  unprimed pulsed at 72–78 h;  $3 \times 10^4$  primed blasts pulsed at 26–36 h;  $3 \times 10^4$  memory cells pulsed at 72–78 h. Before adding 1  $\mu$ Ci [<sup>3</sup>H]TdR to measure T cell proliferation, 50  $\mu$ l of medium was removed for the IL-2 bioassay on  $3 \times 10^4$  Con A blasts in 100  $\mu$ l final volume. The latter values for IL-2 in the medium represent IL-2 release in most cultures. When rIL-2 was added to wells without cells, the level of activity was 30,000 cpm, so that there appeared to be no endogenous IL-2 release in cultures exposed to exogenous IL-2.

One could therefore count the number of DC in the cluster and noncluster fractions and monitor the effect of IL-1 on clustering and on subsequent mitogenesis (see Results).  $1.5\text{--}5 \times 10^4$  dye labeled DC were cultured with  $5 \times 10^6$  CD4<sup>+</sup> cells in 16-mm macrowells for 1 d, and the DC–T aggregates were isolated by velocity sedimentation (18). Aliquots of the clusters were dissociated with gentle pipetting and fixed in 3.7% formaldehyde/PBS. The frequency of fluorescent dye labeled cells was measured in a hemocytometer. The bulk of the clustered cells were returned to culture to assess the mitogenesis that ensued in the presence of known numbers of DC. As will be evident in the Results, all proliferating T cells in the MLR cluster with DC within 24 h of coculture.

## Results

*IL-1 Has No Direct Effect on Unprimed and Sensitized T Lymphocytes.* T lymphocytes in three physiologic states; unprimed cells, freshly sensitized IL-2-responsive blasts, and memory cells were cultured with IL-1, IL-2, IL-1 and IL-2, or DC. IL-1 did not induce either proliferation or IL-2 release, and IL-1 did not synergize with IL-2 (Table I). The IL-1-rich conditioned media of macrophages and keratinocytes (not shown) also had no effect on any of the T cell populations. IL-2 induced significant proliferation only in the T blasts, but no IL-2 release.

TABLE II  
*rIL-1 Does Not Sustain the IL-2 Responsiveness of Helper (Lyt-2<sup>-</sup>) T Blasts*

T cells	Growth (day 1/2)		IL-2 in medium (day 1/2)	
	No rIL-2	500 U/ml rIL-2	No rIL-2	500 U/ml rIL-2
Blasts, rested 2 d, + IL-1	0.2/0.2	0.4/1.3	0.5/0.4	33.9/33.8
Blasts, rested 2 d, no IL-1	0.2/0.3	0.2/1.0	0.3/0.4	36.6/39.8
Freshly prepared lymphoblasts	0.7/0.3	54.1/56.6	0.5/0.3	22.6/14.2

$3 \times 10^5$  alloreactive (C  $\times$  D2 anti-B6.H-2k) Lyt-2<sup>-</sup> T blasts were rested in macroculture with or without 50 U/ml rIL-1 for 2 d.  $3 \times 10^4$  T cell aliquots were then transferred, with or without IL-1 into microtest cultures with graded doses of rIL-2. Proliferation and IL-2 in the medium was then assayed (cpm  $\times 10^{-3}$  as in Table I) at 18 and 42 h (day 1/2).

DC, in contrast, stimulated growth and lymphokine production in all T cell populations (Table I).

To test whether IL-1 could sustain rather than induce IL-2 responsiveness, we cultured IL-2-responsive T blasts for 2 d with or without IL-1, and then added IL-2. Even in the presence of IL-1, the T blasts lost the capacity to respond to IL-2, but not to DC (Table II).

*Lack of Synergism between IL-1 and Antigen or Lectin in Primary Responses.* A long-standing hypothesis states that IL-1 acts in concert with antigen to activate resting lymphocytes. We tested this hypothesis in the primary MLR, where class II MHC products (Ia glycoproteins) carry the antigens recognized by allogeneic CD4 (Lyt-2<sup>-</sup>) T cells. The experiment was made possible by the fact that peritoneal macrophages can be induced to express I-A and I-E products with IFN- $\gamma$ . Expression of Ia was evident on most macrophages by immunofluorescence microscopy (Fig. 1) and by FACS analysis (not shown). These Ia<sup>+</sup> populations induced little or no MLR even in the presence of exogenous IL-1 (Fig. 2). Two other populations of Ia<sup>+</sup> cells, Sephadex G10-nonadherent spleen B cells (17) and UV-inactivated DC were also nonstimulatory, with or without IL-1 (not shown). Therefore, Ia antigens on DC activate unprimed allogeneic T cells, whereas alloantigens on other cells are not stimulatory even in the presence of exogenous IL-1.

We next evaluated whether IL-1 could synergize with polyclonal T cell mitogens. Nylon wool-nonadherent peripheral T cells were treated with anti-Ia and J11d mAb to diminish the level of residual non-T cells that could provide accessory function. The T cells were then cultured with 1  $\mu$ g/ml Con A, exogenous IL-1, and/or accessory cells. The response to mitogen + rIL-1 was minimal, and consistently <5% of a low dose of accessory DC ( $10^3$  DC per  $3 \times 10^5$  T cells; Table III). Similar results were obtained when T cells were modified with another mitogen, sodium periodate (not shown).

*IL-1 Amplifies DC-mediated Responses.* Exogenous IL-1 did amplify the MLR if DC were the source of alloantigen and if IL-1 was added at the onset of culture (Table IV). IL-1 had little or no effect when added at 24 h, or at later times. The amplification of the proliferative response, or the shift in the dose/response curve, varied from 3- to 10-fold. 5 U/ml IL-1 (50 pM) gave maximal amplification, whereas 0.5 U/ml was less than half maximal (not shown).

The effect of IL-1 on DC function was tested in other T cell proliferative

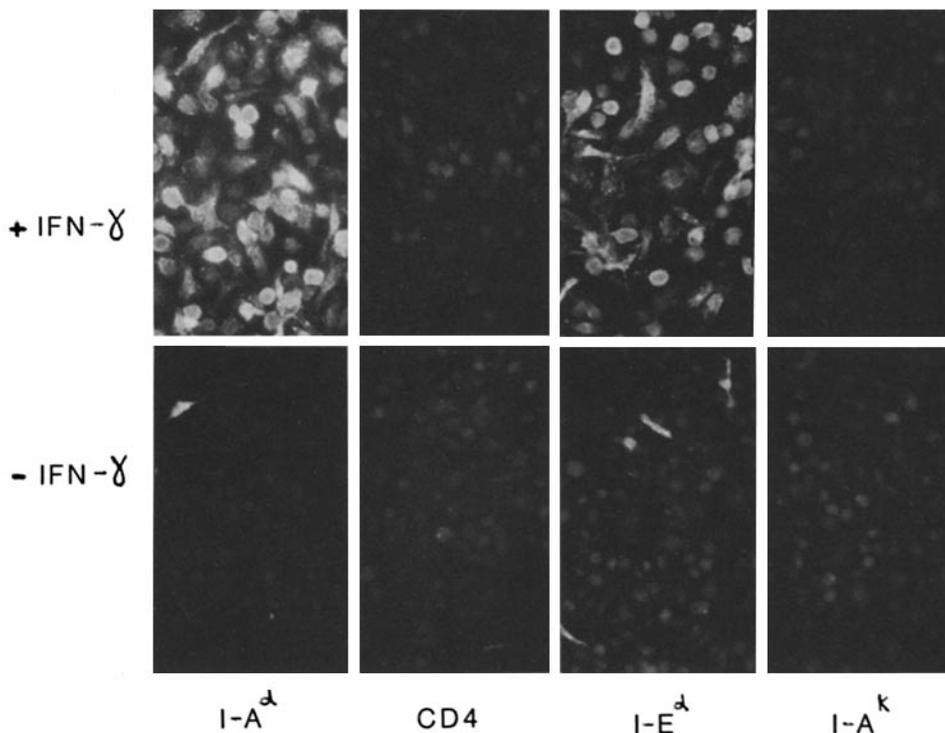


FIGURE 1. Expression of Ia on cultured macrophages. Thioglycollate-elicited, peritoneal cells (>80% macrophages) were cultured with (*top*) or without (*bottom*) IFN- $\gamma$  for 3 d. The cells were maintained as monolayers for 3 d, or in suspension in beakers (32). Shown here are cells from H-2d mice. The micrographs on the left side of the figure were stained (see Materials and Methods) with rat B21-2 anti-I-A<sup>d</sup> and L3T4 (anti-CD4) as the rat IgG2b isotype control. On the right are stains with mouse 14-4-4S anti-I-E<sup>d</sup>, and 10-2.16 anti-I-A<sup>k</sup> as the haplotype and IgG2a isotype control. Note the induction of both I-A and I-E antigens with IFN- $\gamma$  (phase-contrast micrographs are not shown, but >70% of the cells were Ia<sup>+</sup>) in contrast to the low (<3%) frequency of Ia<sup>+</sup> cells in the steady state. Reciprocal results were obtained with B6.H-2k cells in that 10-2.16 stained most of the cells cultured in IFN- $\gamma$ , while B21-2 did not.

responses. Comparable effects were seen in the syngeneic and allogeneic MLR, and in Con A- and periodate-mediated mitogenesis (not shown). The primary antibody response to T-dependent antigens is another model in which DC are active if not essential accessory cells. Addition of rIL-1 increased the efficacy of DC (PFC response per DC) ~10-fold (Table V). In all cases, antibody formation was antigen-dependent and specific (Table V).

*IL-1 Acts on DC Rather than T Cells to Amplify Responses.* We then compared the effects of exogenous IL-1 during the MLR, with prior treatment of the allogeneic DC. Spleen adherent cells were cultured overnight (16 h) with or without 50 U/ml exogenous rIL-1. The cells were washed three times and separated into Fc receptor-negative (EA<sup>-</sup>, DC-enriched) and Fc receptor-positive (EA<sup>+</sup>; 40–50% each, macrophages and B cells, and <10% DC) for use as stimulators of T cell proliferation. The EA<sup>+</sup> fraction, which usually is contaminated with 5–10% DC (29), was <10% as active as the EA<sup>-</sup> fraction. Prior treatment with IL-1 enhanced stimulatory activity especially of the EA<sup>-</sup> fractions

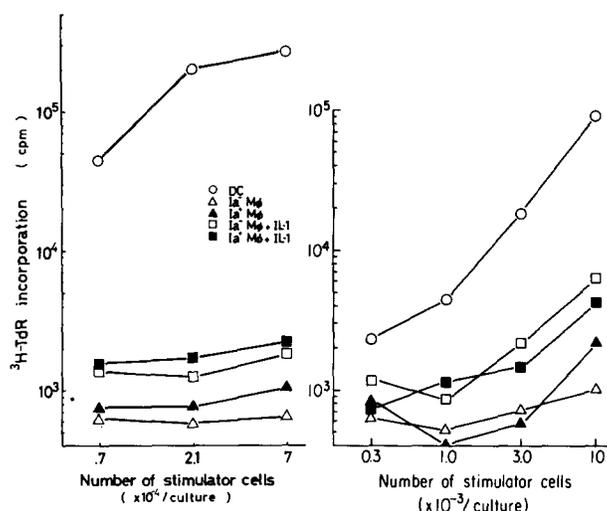


FIGURE 2. IL-1 does not synergize with antigen to activate T cells. Thioglycollate-elicited peritoneal macrophages were cultured with or without IFN- $\gamma$  to yield populations that were >70% I-A<sup>+</sup> and I-E<sup>+</sup> macrophages (Fig. 1). On the left are B6.H-2k cells cultured on coverslips without (Ia<sup>-</sup>) or with (Ia<sup>+</sup>) IFN- $\gamma$ .  $3 \times 10^6$  allogeneic [(C  $\times$  D2)F<sub>1</sub>, H-2d] Lyt-2<sup>-</sup> T cells were added to the coverslips in 16-mm wells, with or without exogenous rIL-1 at 50 U/ml. On the right are (C  $\times$  D2)F<sub>1</sub> peritoneal cells that were cultured in suspension (32) with or without IFN- $\gamma$  and then added to microtest wells with  $3 \times 10^5$  Ia<sup>-</sup>, Lyt-2<sup>-</sup> B6.H-2k T cells, with or without IL-1. At 80–96 h, the cultures were pulsed with [<sup>3</sup>H]TdR to assess the extent of the MLR. Simultaneous experiments showed that the macrophages inhibited the DC-induced MLR 30–70% at the stimulator doses that were used, and that the macrophages were producing little or no endogenous IL-1 (Koide, manuscript in preparation).

TABLE III  
*Lack of Synergism between Con A and IL-1 in the Absence of Accessory Cells*

Additions		Proliferative response (cpm $\times 10^{-3}$ [ <sup>3</sup> H]TdR) of:						
1 $\mu$ g/ml Con A	50 U/ml IL-1	T cells only	T cells + DC			T cells + macrophages		
			30	300	3,000	300	3,000	30,000
–	–	0.2	0.3	0.3	1.0	0.3	0.2	2.0
–	+	0.3	0.3	0.5	0.8	0.3	0.8	2.0
+	–	0.4	1.8	18.3	129	1.1	8.7	26.2
+	+	2.1	5.6	40.5	184	3.0	23.8	45.4

Nylon wool–nonadherent spleen and lymph node lymphocytes were treated with a combination of hybridoma culture supernatants to eliminate Lyt-2<sup>+</sup> cells (TIB 150, anti-Lyt-2) and non-T cells (B21-2 anti-Ia and J11d anti-non-T cell).  $3 \times 10^5$  purified T cells were cultured in flat microtest wells with Con A, IL-1, and accessory cells as indicated. DC were EA<sup>-</sup> low-density spleen adherent cells. Macrophages were 2-h adherent, thioglycollate-elicited peritoneal cells. In the same experiment (not shown), we tested populations of 3-d cultured adherent peritoneal cells (with or without IFN- $\gamma$ ), but these were even less active ( $10^4$  cpm with  $3 \times 10^4$  macrophages and  $2.5 \times 10^3$  cpm with  $3 \times 10^3$  macrophages) than the fresh peritoneal adherent cells.

(Fig. 3). Pulsing DC with IL-1 was as effective as adding IL-1 continuously, and in most experiments (13 of 18) more effective (Fig. 3). The dose of IL-1 that was required to maximally enhance DC function was 5 U/ml (50 pM), and the enhancement increased progressively with exposure time, reaching a plateau at 18–24 h with no further increase after 48 and 72 h (not shown). If the responding

TABLE IV  
*IL-1 Enhances T Cell Proliferative Responses to DC When Added Early in the MLR*

Time of IL-1 addition	Proliferative response (cpm $\times 10^{-3}$ [ $^3\text{H}$ ]TdR) to graded doses DC			
	10,000	3,000	1,000	300
Day 0	77.0 $\pm$ 6.0	40.7 $\pm$ 7.9	21.6 $\pm$ 3.7	16.9 $\pm$ 5.4
Day 1	39.2 $\pm$ 4.6	14.2 $\pm$ 2.9	10.0 $\pm$ 1.5	5.9 $\pm$ 0.8
Day 2	45.1 $\pm$ 7.7	16.2 $\pm$ 3.9	6.2 $\pm$ 2.5	1.2 $\pm$ 0.4
Day 3	35.4 $\pm$ 1.1	10.4 $\pm$ 2.6	4.6 $\pm$ 1.4	1.7 $\pm$ 0.3
No IL-1	37.4 $\pm$ 4.1	8.9 $\pm$ 2.5	2.4 $\pm$ 0.7	2.4 $\pm$ 0.9

Graded doses of H-2<sup>d</sup> DC were added to purified H-2k T cells ( $3 \times 10^5$ ). IL-1 was omitted from the cultures or added at 50 U/ml at the indicated times. Data are means of triplicates  $\pm$  SD for MLRs at 72–90 h. T cells only gave  $1.2 \times 10^3$  cpm uptake.

TABLE V  
*Effects of rIL-1 and DC on Primary T-dependent Antibody Responses*

Exp.	Cells	Antibody response (anti-TNP/anti-SRC PFC) with:					
		No IL-1, and:			50 U/ml IL-1, and:		
		No antigen	TNP-KLH	SRC	No antigen	TNP-KLH	SRC
1	$4 \times 10^6$ B/T	0/4	0/4	8/0	4/0	4/0	0/8
	B/T + $3 \times 10^4$ DC	4/0	1,116/144	16/716	12/8	1,360/260	132/1,008
	B/T + $10^4$ DC	12/4	980/32	20/544	12/8	1,616/300	76/992
	B/T + $3 \times 10^3$ DC	12/4	504/20	4/432	8/4	1,408/256	92/784
	B/T + $10^3$ DC	4/0	352/4	0/188	4/4	1,024/200	68/500
	B/T + $3 \times 10^2$ DC	0/8	76/0	0/84	8/4	652/84	32/348
	B/T + $10^2$ DC	4/0	16/0	0/28	8/0	348/16	4/220
2	$4 \times 10^6$ B/T	0/0	20/0	0/0	8/12	140/24	20/16
	$2 \times 10^6$ B	NT	NT	NT	4/0	28/12	0/0
	B + $10^4$ DC	NT	NT	NT	0/0	16/8	4/0
	B/T + $3 \times 10^4$ DC	20/4	1,540/128	20/916	NT	NT	NT
	B/T + $10^4$ DC	4/0	1,032/132	0/456	56/40	1,352/108	4/716
	B/T + $3 \times 10^3$ DC	0/4	800/96	12/204	12/0	1,188/124	4/584
	B/T + $10^3$ DC	0/0	500/68	20/56	28/0	1,092/112	4/428

Macrocultures (1 ml medium 5% FCS in RPMI 1640 with  $5 \times 10^{-5}$  M 2-ME; 16-mm-diam wells) were set up with the indicated numbers of B/T (Sephadex G10–nonadherent spleen), B (G10–nonadherent treated with anti-Lyt-1, anti-Thy-1, and complement), and DC (low-density spleen adherent cells depleted of macrophages by readherence to plastic). The antigens were none, 5  $\mu\text{g}/\text{ml}$  TNP-KLH, or  $2 \times 10^6$  SRBC. After 4 d, plaque assays were run on TNP-SRC or SRBC indicator cells.

T cells were pulsed with IL-1 or if allogeneic Ia<sup>+</sup> macrophages were pulsed, there was no enhancement of the subsequent MLR (not shown).

*Other Cytokines Do Not Enhance the Accessory Function of DC.* Several recombinant cytokines and two conditioned media that were rich in natural IL-1 were added to DC for 18 h. The cells were washed, and the EA<sup>-</sup> DC were used as stimulators of T cell mitogenesis. Only the exposure to IL-1, but not to other lymphokines further enhanced DC function for all four T cell proliferative

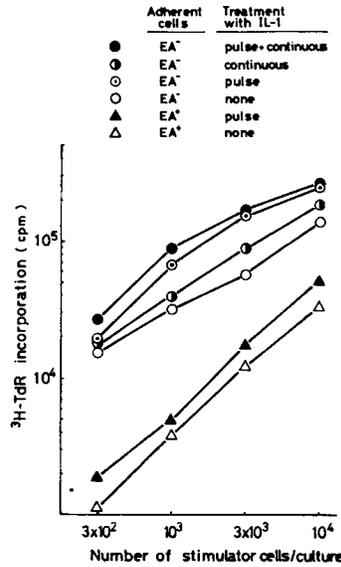


FIGURE 3. Effect of rIL-1 on DC function in the MLR. Low-density spleen adherent cells were cultured overnight with or without 50 U/ml IL-1, washed, and separated into EA<sup>-</sup> and EA<sup>+</sup> fractions. The cells were added in graded doses to 3 × 10<sup>5</sup> allogeneic T cells with or without additional exogenous IL-1, as indicated. The MLR was measured at 72–90 h. Note that DC that were pulsed with IL-1 were some 3–10 times more effective.

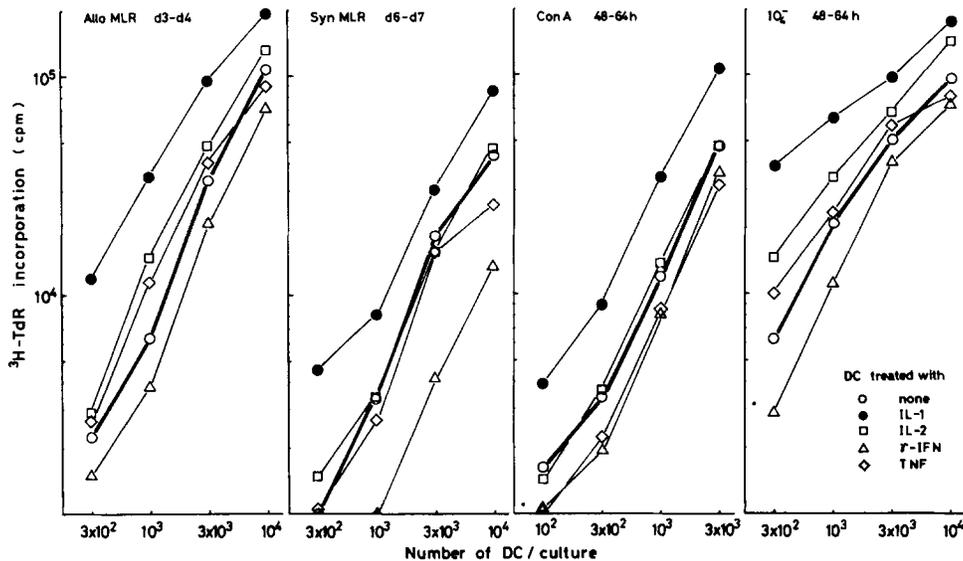


FIGURE 4. Effect of IL-1 on DC function in T cell mitogenesis. Low-density spleen adherent cells were cultured overnight in the presence of recombinant cytokines. The cells were washed, and EA<sup>-</sup> DC were prepared and added to purified T cells in four different proliferative assays. In all cases, pulsing the DC with IL-1 enhanced function while pulsing with IFN-γ decreased function.

TABLE VI  
Pulsing with IL-1, But Not with Other Cytokines, Enhances the Accessory Function of DC

Cytokine/dose	Proliferative response (cpm $\times 10^{-3}$ [ $^3\text{H}$ ]TdR) to graded doses of DC					
	MLR (day 3-4)			Con A (day 2-3)		
	10,000	3,000	1,000	1,000	300	100
None	71.9 $\pm$ 11.7	15.8 $\pm$ 3.3	2.7 $\pm$ 0.8	71.7 $\pm$ 8.0	28.6 $\pm$ 0.9	11.5 $\pm$ 1.5
rIL-1, 50 U/ml	150.4 $\pm$ 5.6	47.0 $\pm$ 1.6	9.7 $\pm$ 0.7	130.6 $\pm$ 13.4	68.7 $\pm$ 0.4	35.8 $\pm$ 3.8
30% Keratinocyte supernatant	142.5 $\pm$ 11.3	38.3 $\pm$ 2.0	7.9 $\pm$ 1.4	134.2 $\pm$ 2.7	64.4 $\pm$ 3.4	33.1 $\pm$ 2.6
30% Macrophage supernatant	152.2 $\pm$ 11.6	42.8 $\pm$ 6.9	8.0 $\pm$ 1.5	148.2 $\pm$ 10.2	69.2 $\pm$ 6.0	29.4 $\pm$ 1.3
rIL-2, 100 U/ml	102.1 $\pm$ 8.2	18.0 $\pm$ 1.5	5.2 $\pm$ 0.7	95.3 $\pm$ 6.9	37.7 $\pm$ 2.6	14.9 $\pm$ 1.2
30% WEHI-3 supernatant	104.2 $\pm$ 2.7	21.7 $\pm$ 5.1	3.6 $\pm$ 0.2	94.3 $\pm$ 7.1	39.6 $\pm$ 1.0	17.2 $\pm$ 2.2
rIFN- $\gamma$ , 100 U/ml	15.0 $\pm$ 1.6	2.1 $\pm$ 0.3	0.4 $\pm$ 0	42.6 $\pm$ 2.6	14.8 $\pm$ 2.5	7.3 $\pm$ 1.2
rTNF, 1 ng/ml	86.4 $\pm$ 7.3	17.2 $\pm$ 2.1	2.7 $\pm$ 0.6	94.9 $\pm$ 4.1	40.9 $\pm$ 2.6	15.1 $\pm$ 2.8

$3 \times 10^5$  Ia<sup>-</sup>, Lyt-2<sup>-</sup> T cells were stimulated with graded doses of allogeneic DC (MLR) or syngeneic DC (Con A) that had been pulsed overnight with the indicated cytokines. The data are proliferative responses  $\pm$  SD. IL-1 activities (D10 assay) for the keratinocyte- and macrophage-conditioned media were 5 and 10 U/ml respectively. T cell only responses were 748 and 5,671 cpm for the MLR and lectin responses.

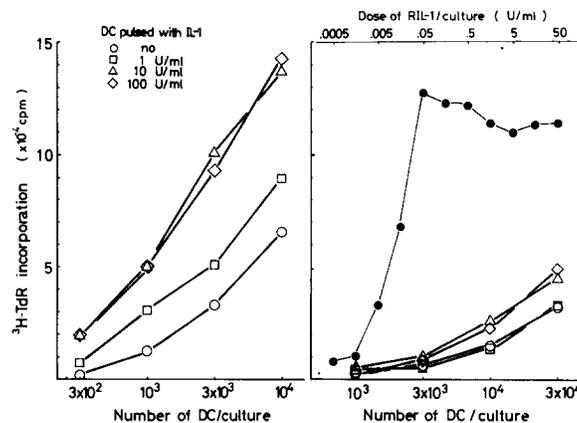
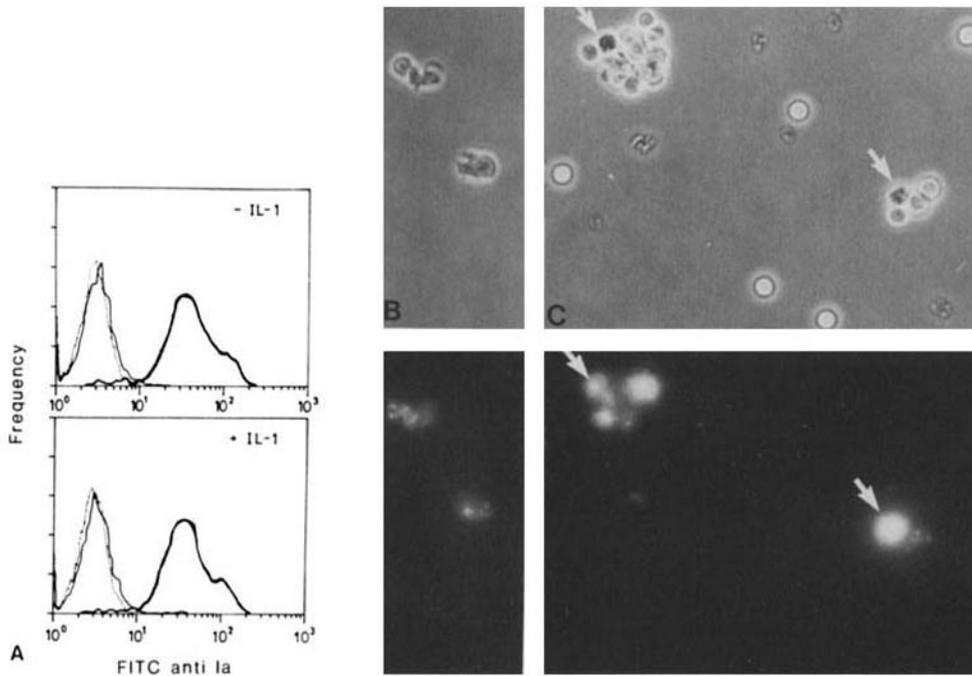


FIGURE 5. Effects of IL-1-pulsed DC on the MLR and the D10 cell line. DC were cultured overnight in the indicated doses of IL-1 and then added in graded doses to allogeneic CD4<sup>+</sup> T cells (*left*) or to the IL-1-responsive D10 cell line (*right*). The effects of graded doses of rIL-1 on the cell line are shown (*filled circles*). Note that a dose of 1 U/ml is required to detect an increase in DC function in the MLR, but that DC exhibit little or no IL-1 activity in the D10 bioassay.

responses (Fig. 4). The effects of macrophage and keratinocyte culture media were similar to rIL-1 (Table VI). IL-2, IL-3, and cachectin/TNF had little effect, while exposure to IFN- $\gamma$  decreased DC function in both experiments (Fig. 4, Table VI).

*Studies on Mechanism of Action of IL-1 on DC.* To test whether IL-1-pulsed DC were simply carrying over this cytokine into the subsequent culture, we added DC that had been exposed to graded doses of IL-1 to the MLR and D10 assays. Exposure of DC to 1 U/ml IL-1 produced a detectable increase in DC function in the MLR, while 10 U/ml gave a maximal effect (Fig. 5, *left*). However, DC that had been pulsed with 0–100 U/ml IL-1 had comparable but weak IL-1 activity in the sensitive D10 bioassay (Fig. 5, *right*). The activity of DC at the highest dose used for accessory function (30,000 DC) corresponded to 0.01 U/ml of exogenous IL-1, yet 1 U/ml was the minimal continuous dose needed



**FIGURE 6.** Studies of the effects of IL-1 on DC. (A) Effect of IL-1 on expression of Ia antigens. After 18 h of culture with or without 50 U/ml of rIL-1, DC were exposed to FITC-B21-2 (*thick line*), or FITC-goat anti-mouse Ig (*thin line*), or FITC-9.3 (anti-human Ia; *dashed line*) and analyzed on the FACS. Note that the level of Ia on DC was not changed by IL-1. The amount of Ia on DC was about twice that observed on macrophages exposed to IFN- $\gamma$ , as was previously documented using binding of <sup>125</sup>I-B21-2 (22). (B) Carbocyanine-dye labeling of DC. Carbocyanine dyes are stable nontoxic labels that permit the enumeration of DC in long-term culture. Shown are two DC immediately after labeling with 20  $\mu$ g/ml diI and observed under the hemocytometer by phase contrast (*top*) or fluorescence (*bottom*). The labeling consists of a diffuse fluorescence as well as a number of bright cytoplasmic granules. (C) Movement of dye-labeled DC into T cell clusters. DC, labeled as in B, were added to allogeneic CD4 cells for 1 d. Cluster (shown here) and noncluster fractions were isolated and the fluorescent DC (two labeled cells, shown by arrow) counted on a hemocytometer (see Table VII for data).

to see an enhancing effect in the MLR. Also, the MLR-stimulating activity of 300 DC was clearly enhanced by prior exposure to IL-1 (Fig. 5, *left*), but this dose of DC had little or no effect in the IL-1 bioassay. In other experiments (not shown), IL-1-pulsed DC were UV irradiated to ablate accessory function, and were added to control DC. No amplification of DC function was observed (not shown). We conclude that the enhanced activity of IL-1-pulsed DC is not the result of carryover of IL-1 on or with DC. Instead, exposure to IL-1 enhances subsequent DC function.

When exogenous IL-1 was added as a pulse or continuously to DC, viable cell recovery was not altered if examined at daily intervals for 72 h. Expression of cell surface Ia was not changed (Fig. 6). However, IL-1-pulsed DC clustered more effectively with T cells in the MLR, and this enhanced clustering was sufficient to account for the increase in accessory function (Table VII). To obtain

TABLE VII  
*IL-1-treated DC Cluster More Efficiently with Allogeneic CD4<sup>+</sup> T Lymphocytes*

Exp.	Culture conditions*		Distribution of DC at 24 h (specific activity and percent recovery) <sup>‡</sup>		Proliferation (cpm × 10 <sup>-3</sup> [ <sup>3</sup> H]TdR) <sup>§</sup>		
	Initial DC/T ratio	IL-1 pulse of DC	Clusters	Nonclusters	Clusters	Non-clusters	Mix
1	1:300	No	0.7 (46)	0.08 (54)	1.3	0.4	1.9
	1:300	Yes	1.7 (82)	0.04 (18)	11.7	0.5	14.6
	1:100	No	2.3 (33)	0.32 (67)	10.5	0.6	19.4
	1:100	Yes	4.8 (65)	0.23 (35)	59.0	1.0	87.2
2	1:300	No	1.9 (61)	0.06 (39)	11.9	0.3	16.1
	1:300	Yes	3.6 (71)	0.06 (29)	35.4	0.4	63.8
	1:100	No	7.1 (49)	0.28 (51)	69.8	0.4	88.2
	1:100	Yes	9.5 (57)	0.33 (43)	97.0	0.5	101.5

\* DC were cultured for 18 h with or without 50 U/ml rIL-1 and then labeled with 20 µg/ml carbocyanine dye. The DC were then added to 5 × 10<sup>6</sup> CD4<sup>+</sup> (Lyt-2<sup>-</sup>) T cells for 24 h.

<sup>‡</sup> At 24 h of the MLR, clustered and nonclustered fractions were separated by velocity sedimentation, and aliquots were counted in a hemocytometer for total cells and dye-labeled DC. Specific activity means percent DC in that fraction; recovery means percentage of total DC that were recovered from the velocity gradient in that fraction. The yields of DC and T cells from the columns corresponded to 50% of the cells that were added initially to the culture. The yield of clustered cells was identical in all cultures and corresponded to 5–10% of the cells recovered from the Percoll gradient.

<sup>§</sup> Isolated clustered and nonclustered fractions were returned to culture to follow cell proliferation in the presence of known numbers of DC. Data are means of triplicates pulsed on day 3. The cultures contained 3 × 10<sup>4</sup> clustered cells, 2 × 10<sup>5</sup> nonclustered cells, or mixtures thereof. In Exp. 2, we also monitored proliferation at 26–34 h, i.e., just after isolation of the clusters. The values were 1.3–9.8 × 10<sup>5</sup> for clusters and 0.4–1.8 × 10<sup>5</sup> for nonclusters, indicating that DNA synthesis was just beginning at the time we measured the number of clustered DC.

this information, we labeled the DC with a carbocyanine dye, which provided a stable, nontoxic, fluorescent probe (31 and Fig. 6, *middle*). Movement of labeled DC into DC–T cell clusters could then be monitored by fluorescence microscopy (Fig. 6 *right*) and was found to be directly proportional to DC dose (Table VII). IL-1 pretreatment enhanced the number of clustered DC but not T cells (Table VII). When mitogenesis was monitored by [<sup>3</sup>H]TdR uptake, the T cells proliferated in accordance with DC numbers (regression coefficient of 0.98; not shown) whether or not the DC had been cultured with IL-1 (Table VII). We conclude that IL-1 enhances the capacity of DC to cluster responsive T cells before the onset of DNA synthesis, but that IL-1 probably does not change the function of DC or T cells once clustering has occurred.

### Discussion

DC are active if not essential accessory cells for the growth of helper T lymphocytes. To understand their mechanism of action, we are testing whether lymphocyte-activating factors like IL-1 replace the function of DC, and whether DC release these factors. In this paper, we have compared the effects of DC with exogenous murine rIL-1α, which was the first activating factor to become available in substantial quantity and purity. Elsewhere we will describe our

experiments showing that DC do not produce endogenous IL-1 when stimulated with such agents as LPS (Koide, S. L. and R. M. Steinman, manuscript submitted for publication).

Using several populations of peripheral T cells and mitogenesis assays, we cannot find support for the idea that IL-1 can mimic the function of DC. Exogenous IL-1 does not directly influence the proliferation of isolated T cells in mitogen or MLR systems (Tables I and II), and does not synergize with mitogen or with antigen (Table III and Fig. 2). In contrast, small doses of DC are markedly stimulatory. When neutralizing antibodies to IL-1 are available, it will be important to test whether these antibodies interfere with DC function. However, the view that active accessory cells like DC are simply a source of antigen plus IL-1 does not seem to be correct. There may yet be subsets of T cells that directly use IL-1 during lymphocyte growth. The D10 line that provides a sensitive IL-1 bioassay is an example of a T cell that seems to grow in response to IL-1 but not to DC (Fig. 5).

While exogenous IL-1 does not act as a lymphocyte-activating factor, we have discovered that low concentrations (plateau of 50 pM) significantly amplify responses to limiting doses of DC (Tables III–V; Fig. 3). The enhancing effect can be reproduced if the DC but not the T cells are exposed to the cytokine before use as accessory cells (Table VI; Figs. 3–5). The phenomenon seems to be specific for IL-1 rather than other cytokines, including cachectin/TNF, which shares several functional properties with IL-1 (31). It seems unlikely that lipopolysaccharide-induced release of endogenous IL-1 might contribute to the basal stimulatory function of DC. We have observed normal levels of DC function in two experiments with LPS-unresponsive, C3H/HeJ mice. Also we typically do not find endogenous IL-1 production in spleen adherent cells (Koide and Steinman, manuscript submitted for publication).

Our studies of mechanism indicate that IL-1 must be applied to the DC for some 18 h for maximal effect, and that there is no carryover of active IL-1 into the culture (Fig. 5). Nor does IL-1 increase the level of Ia on DC (Fig. 6). The one alteration in DC function that we can pinpoint is the more efficient clustering of IL-1 treated DC with T cells before the onset of mitogenesis (Fig. 6, Table VII). Once clustered, control and IL-1-treated DC seem equally efficient in inducing a proliferative response. IL-1 is the first molecule to be identified that enhances immunity by enhancing DC function.

### Summary

The function of exogenous murine recombinant IL-1 $\alpha$  as a T lymphocyte-activating molecule was examined. IL-1 did not induce IL-2 release or responsiveness in purified T cells regardless of their state of activation: unprimed lymphocytes, freshly sensitized lymphocytes, or memory cells derived from the blasts. Nor did IL-1 synergize with mitogens, or with antigens, to stimulate proliferation. For example the combinations of IL-1 plus Ia<sup>+</sup> peritoneal macrophages, or IL-1 plus Con A, were <5% as effective in triggering T cell growth as a low dose (1%) of dendritic cells. However, when IL-1 was added at the onset of culture, the response to limiting doses of dendritic cells was increased 3- to 10-fold in several systems: the syngeneic and allogeneic MLR, Con A- and

periodate-induced polyclonal mitogenesis, and T-dependent antibody formation against foreign red cells.

The amplifying effect of IL-1 could be obtained if the dendritic cells but not the responding lymphocytes were exposed to IL-1 before use as accessory cells. Optimal activation of dendritic cells required a dose of 5 U/ml (50 pM) and 18 h of exposure, and was not due to carryover of IL-1 into the lymphocyte culture. IL-2, IL-3, and cachectin/TNF did not amplify dendritic cell function, while IFN- $\gamma$  diminished it. The enhanced function of IL-1-treated dendritic cells was due to an enhanced clustering with helper T lymphocytes in the first day of the MLR response.

Therefore IL-1 does not seem to act as an activating factor for most peripheral T lymphocytes. Instead, IL-1 enhances the function of accessory dendritic cells and represents the first molecule that has been shown to enhance the immune response at this critical level.

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